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(54) Title: ALKALINE PROTEOLYTIC ENZYME AND METHOD OF PRODUCTION

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                    5        10       15       20       25       30
                    |        |        |        |        |        |
       1 M K K P L G K I V A S T A L L I S V A F S S S I A S A A E E
      31 A K E K Y L I G F N E Q E A V S E F V E Q V E A N D E V A I
      61 L S E E E E V E I E L L H E F E T I P V L S V E L S P E D V
      91 D A L E L D P A I S Y I E E D A E V T T M A Q S V P W G I S
     121 R V Q A P A A H N R G L T G S G V K V A V L D T G I S T H P
     151 D L N I R G G A S F V P G E P S T Q D G N G H G T H V A G T
     181 I A A L N N S I G V L G V A P S A E L Y A V K V L G A D G R
     211 G A I S S I A Q G L E W A G N N G M H V A N L S L G S P S P
     241 S A T L E Q A V N S A T S R G V L V V A A S G N S G A S S I
     271 S Y P A R Y A N A M A V G A T D Q N N N R A S F S Q Y G A G
     301 L D I V A P G V N V Q S T Y P G S T Y A S L N G T S M A T P
     331 H V A G A A A L V K Q K N P S W S N V Q I R N H L K N T A T
     361 S L G S T N L Y G S G L V N A E A A T R


Amino acid composition

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       54 A      0 C       8 H       5 M      21 T
        9 R     12 Q      19 I       6 F       3 W
       24 N     28 E      30 L      17 P       9 Y
       11 D     36 G      10 K      43 S      36 V

Number of residues:  380
Molecular weight (MW):  38951
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(57) Abstract

A proteolytic enzyme for use in detergent formulations having increased pH and oxidative stability under typical laundering conditions in aqueous solutions is produced by fermenting *Bacillus licheniformis* host strain transformed by a multicopy plasmid comprised of DNA sequences which code for the desired proteolytic enzyme.

* See back of page

DESIGNATIONS OF "DE"

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ALKALINE PROTEOLYTIC ENZYME AND METHOD OF PRODUCTION

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to an alkaline proteolytic enzyme, its production by cultivating genetically modified Bacillus strains, and the genetic constructs within the

5   Bacillus strains which code for the alkaline proteolytic enzyme.

2. Description of the Related Art

Proteolytic enzymes are widely used in household laundry detergent formulations to remove protein-based

10   stains from articles of clothing and the like. These enzymes are members of the class known as serine proteases, enzymes which catalyze the cleavage of peptide bonds. To be useful in aqueous detergent solutions, these enzymes must be active at pH values ranging from 7 to 10. They

15   must also be stable to oxidation and enzymatic and non-enzymatic hydrolysis in order to be useful in detergent applications. Various Bacillus strains produce serine proteases under suitable aerobic fermentation conditions. However, the amount of protease made by the wild-type

20   strains and the rate of production of the protease is

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usually insufficient for commercial production. One method
of increasing the protease yield is to generate mutant
strains through exposure to radiation or chemical mutagens.
However, these classical mutation processes suffer from the
5 disadvantage that they produce only a statistical
distribution of the desired mutation and/or that a
significant population reverts back to the wild type. In
recent years, efforts to overcome these problems have
centered on directed genetic modification of bacterial
10 strains. This is accomplished through the introduction of
recombinant DNA into host cells under circumstances
guaranteeing that the host cells will replicate the
recombinant DNA with an increased copy number. In
addition, the genes carried by the foreign DNA must be able
15 to be expressed. The genetically engineered organisms do
not suffer from the disadvantages often exhibited by
classically mutagenized bacteria and are, therefore, often
useful for commercial enzyme production.

Methods of making proteases using recombinant DNA
20 technology are known. For example, European Patent
EP130756A discloses a method for preparing carbonyl
hydrolase (a protease), U.S. patent No. 4,760,025 discloses
a subtilisin from having an altered amino acid sequence
than that produced by the naturally occuring strain.
25 German patent No. DE 2925427 discloses a <u>Bacillus</u> alkaline
protease which is highly stable to chelating agents and
surfactants, and is produced by fermentation of a <u>Bacillus</u>
<u>licheniformis</u> strain. Production of an alkaline proteolytic
enzyme by cultivation of <u>B</u>. <u>licheniformis</u> has been
30 described in British patent No. 1,263,765; German patent
No. 1,940,488; Russian patent No. 400,116; U.S. patent
Nos. 3,623,956; 3,623,957.

<u>SUMMARY OF THE INVENTION</u>

It is an object of the present invention to provide a
35 proteolytic enzyme for use in detergent formulations having
increased protease and oxidative stability under conditions
of pH 7-10 and at temperatures of 10-60°C in aqueous

solutions. It is also an object of the present invention to provide mutant *Bacillus* strains for use in the commercial production of proteolytic enzymes useful in detergent formulations. It is a further object of the present

5 invention to provide expression vectors containing sequences which encode the desired proteolytic enzyme for transforming host *Bacillus* cells. These objectives are accomplished by providing a mature proteolytic enzyme having the amino acid sequence substantially as shown in

10 Figure 1. The mature proteolytic enzyme has the amino acid sequence which extends from about amino acid residue number 112 to about amino acid residue number 380. This proteolytic enzyme is obtained by cultivating a *B. licheniformis* ATCC 53926 strain which has been transformed

15 by a plasmid having a DNA sequence comprising in the direction of transcription a regulatory region, said regulatory region comprising in the order of transcription a *B. licheniformis* ATCC 53926 alkaline protease gene promoter, ribosome binding site, and initiation codon

20 operably linked to a pre-pro region, said pre-pro region operably linked to a mature region encoding for the *Bacillus lentus* alkaline protease enzyme.

The strain *Bacillus licheniformis* ATCC 53926 will hereinafter be referred to as ATCC 53926. The strain

25 *Bacillus licheniformis* DSM 641 will hereinafter be referred to as DSM 641. The Polymerase Chain Reaction which is described in U.S. patent Nos. 4,683,195 and 4,683,202 will hereinafter be referred to as PCR. The *Bacillus licheniformis* ATCC 53926 alkaline protease gene, shown in

30 Figure 27, will hereinafter be referred to as ATCC 53926 alkaline protease gene. *Bacillus lentus* alkaline protease will hereinafter be referred to as BLAP. The *Bacillus licheniformis* ATCC 53926 alkaline protease gene promoter is the promoter from the ATCC 53926 alkaline protease gene and

35 will hereinafter be referred to as the ATCC 53926 alkaline protease gene promoter. The abbreviation kb will hereinafter be used in place of 1000 base pairs.

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BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the amino acid sequence for the pre, pro, and mature regions of the BLAP protein.

Figure 2 shows the DNA sequence for the 3.4 kb HindIII fragment from Bacillus lentus which includes the BLAP gene.

Figure 3 shows the restriction map of plasmid pGM15.

Figure 4 shows the restriction map for plasmid pCB11C.

Figure 5 shows the restriction map for plasmid pCB1N.

Figure 6 shows the restriction map for plasmid pCB13C.

Figure 7 shows the restriction map for plasmid pCB2N.

Figure 8 shows the oligonucleotides 1-3. Positions correspond to the positions of the nucleotide sequences within the ATCC 53926 alkaline protease gene. Mismatch positions are indicated by the use of lower case letters. Restriction sites are underlined and indicated.

Figure 9 shows the restriction map for plasmid pCB15C.

Figure 10 shows the restriction map for plasmid pCB4N.

Figure 11 shows a scheme for the construction of plasmid pCB1N.

Figure 12 shows a scheme for the construction of plasmid pCB11C.

Figure 13 shows the restriction map for plasmid pCB55C.

Figure 14 shows the restriction map for plasmid pCB56C.

Figure 15 shows the restriction map for plasmid pCB58C.

Figure 16 shows the restriction map for plasmid pCB75C.

Figure 17 shows the restriction map for plasmid pCB78C.

Figure 18 shows the restriction map for plasmid pCB50N.

Figure 19 shows the restriction map for plasmid pCB51N.

Figure 20 shows the restriction map for plasmid pCB53N.

Figure 21 shows the restriction map for plasmid pCB70N.

Figure 22 shows the restriction map for plasmid pCB73N.

Figure 23 shows the scheme for construction of the ClaI fusion plasmid constructs in Escherichia coli and Bacillus.

Figure 24 shows a schematic diagram of the Savinase oligonucleotide probes. These probes were designed on the basis of known Savinase amino acid sequence to probe the

<u>Bacillus</u> <u>lentus</u> gene banks and to serve as primers in the PCR reaction.

Figure 25 shows the specific sequence of oligonucleotide probes and primers.

Figure 26 shows the restriction map for plasmid pMG108.2

Figures 27 and 27a show the introduction of restriction sites into the gene encoding the ATCC 53926 alkaline protease gene.

Figure 28 shows a comparison of the relative protease yields of the different ATCC 53926 alkaline protease-BLAP constructs in the ATCC 53926 production strain.

Figure 29 shows the amino acid sequence of the mature protease made from the ATCC 53926 alkaline protease-BLAP <u>Nco</u>I gene fusion constructs.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

A proteolytic enzyme (BLAP) produced by <u>B</u>. <u>lentus</u>, is provided having increased protease and oxidative stability in aqueous solutions of pH 7-10 and at temperatures of from 10-60°C. The mature BLAP enzyme is a polypeptide having 269 amino acid residues, a molecular weight of 26,823 daltons, and a calculated isoelectric point of 9.7. It was first obtained from <u>B</u>. <u>lentus</u> DSM 5483, an organism isolated from soil samples and having the properties listed in Table I.

Table I. Properties of <u>B</u>. <u>lentus</u> DSM 5483

TAXONOMIC NAME	<u>B</u>. <u>lentus</u> (Group II)
MORPHOLOGY	Vegetative Cells:
	Rods
	Width 0.6-0.8 μm
	Length 1.5-3.5 μm
	Spores:
	Ellipsoid
	Sporangium not swollen
MOBILITY	Motile
GRAM REACTION	Positive
CATALASE	Positive
ANAEROBIC GROWTH	Negative

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VOGUES-PROSKAUER REACTION	Negative		

pH in Vogues-Proskauer medium: 6.2

GROWTH PARAMETERS

TEMPERATURE

Growth	50°C
No growth	55°C
Optimal	39°C

pH:

Growth	pH 7.5 - pH 9.5
No growth	pH 5.7

SALT TOLERANCE

Grows in   5% NaCl
Grows in   7% NaCl
Grows in   10% NaCl

SUITABLE MEDIUM:

Potato Starch	10	g/l	
Yeast Extract	10	g/l	
Peptone	5	g/l	
$Na_2S_2O_3 \cdot 5H_2O$	2.5	g/l	
K_2HPO_4	2	g/l	
$Na_2MoO_4 \cdot 2H_2O$	1.2	g/l	
NH_4Cl		1	g/l
$FeCl_3 \cdot 6H_2O$		540	mg/l
MgO	500	mg/l	
$CaCO_3$		200	mg/l
$MnCl_2 \cdot 4H_2O$		100	mg/l
ZnO	40	mg/l	
$CoCl_2 \cdot 6H_2O$		24	mg/l
$CuCl_2 \cdot 2H_2O$		20	mg/l
H_3BO_4		3	mg/l

ACID PRODUCTION FROM:	Glucose	(weak)
	L-Arabanose	(weak)
	Xylose	(weak)
	Mannitol	(weak)

GAS PRODUCTION FROM GLUCOSE   Negative

LECITHINASE   Negative

HYDROLYSIS	Starch:	Positive
	Gelatin:	Positive
	Casein:	Positive

ACID UTILIZATION	Citrate:	Positive
	Propionate:	Negative

TYROSINE DEGRADATION   Negative

INDOLE REACTION   Negative

PHENYLALANINE DEAMINASE   Negative

ARGININE DEHYDROLASE   Negative

5 NITRATE REDUCTION   Nitrate reduced to nitrite

   The amino acid sequence, shown in Figure 1 was
determined in part by standard amino acid sequencing
techniques, and the rest deduced from the nucleic acid
10 sequence.

   A method is presented for producing the BLAP enzyme
comprising cultivating a Bacillus host cell containing a
hybrid plasmid having a DNA sequence coding for the BLAP
enzyme comprising in the direction of transcription, a
15 regulatory region, said regulatory region comprising in the
order of transcription a ATCC 53926 alkaline protease gene
promoter, ribosome binding site, and initiation codon
operably linked to a pre-pro region, said pre-pro region
operably linked to a mature region encoding for the BLAP
20 enzyme. All the above may be linked to a carboxy terminal
(C-terminal) DNA sequence from ATCC 53926 alkaline protease
gene. The C-terminal DNA sequence is a DNA sequence
between approximately the SalI and approximately the SstI
restriction sites of the ATCC 53926 alkaline protease gene.
25 It may be used to suppress chromosomally encoded protease
as disclosed in a copending U.S. patent application. Any
Bacillus strain can be used in the method of the present
invention to produce the BLAP enzyme. B. licheniformis is
the preferred strain. ATCC 53926 is the most preferred
30 strain. ATCC 53926 is a mutant strain obtained by classical
mutagenesis of B. licheniformis DSM 641 having the
properties listed in Table II.

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Table II. Properties of <u>B</u>. <u>licheniformis</u> ATCC 53926

TAXONOMIC NAME	<u>B</u>. <u>licheniformis</u> ATCC 53926	
MORPHOLOGY	Vegetative Cells:	
	Rods	
5	Width	0.7-0.9 μm
	Length	2.0-3.0 μm
	Spores:	
	Ellipsoid	
10	Sporangium not swollen	
MOBILITY	Motile	
GRAM REACTION	Positive	
15		
CATALASE	Positive	
ANAEROBIC GROWTH	Positive	
20 VOGUES-PROSKAUER REACTION	Positive	
	pH in Vogues-Proskauer medium: 5.5	
GROWTH PARAMETERS		
	TEMPERATURE	
25	Growth	50°C
	No growth	55°C
	pH:	
	No growth	pH 5.7
30	SALT TOLERANCE	
	Grows in 5% NaCl	
	Grows in 7% NaCl	
	Grows in 10% NaCl	
35 ACID PRODUCTION FROM:	Glucose	positive
	L-Arabanose	positive
	Xylose	positive
	Mannitol	positive
40 GAS PRODUCTION FROM GLUCOSE		Negative
LECITHINASE		Negative
HYDROLYSIS	Starch:	Positive
45	Gelatin:	Positive
	Casein:	Positive
ACID UTILIZATION	Citrate :	Positive
	Propionate:	Negative
50		
TYROSINE DEGRADATION	Negative	
INDOLE REACTION	Negative	
55 PHENYLALANINE DEAMINASE	Negative	

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ARGININE DEHYDROLASE   Negative

NITRATE REDUCTION   Nitrate not reduced to nitrite

5   The gene coding for the BLAP enzyme can be obtained by
isolating the chromosomal DNA from the B. lentus strain DSM
5483, constructing DNA probes having homology to putative
DNA sequences encoding regions of the B. lentus protease,
preparing genomic libraries from the isolated chromosomal
10   DNA, and screening the libraries for the gene of interest
by hybridization to the probes. The genomic libraries can
be constructed in E. coli using plasmid pBR322,
bacteriophage lambda EMBL3 and EMBL4, and the cosmid pCP13
as cloning vectors. Preferably, the gene bank of B. lentus
15   DSM 5483 chromosomal DNA is constructed in E. coli with
the cosmid pCP13.

       Oligonucleotide probes having DNA sequences similar to
those of the Savinase gene (a gene encoding for Savinase,
a commercial serine protease sold by Novo Industri A/S) can
20   be used to probe for Savinase-like genes. Preferably, a
BLAP sequence can be used. These oligonucleotides can be
used to screen Southern blots of restriction enzyme
digested chromosomal DNA's from E. coli HB101, B. subtilis
DB104, B. licheniformis, and the B. lentus strain. The N-
25   terminal and internal oligonucleotides as shown in Figures
24 and 25 can also be utilized as primers in the PCR to
amplify that region of B. lentus chromosomal DNA extending
from the N-terminus of the mature alkaline protease to the
N-terminus of the C-terminal cyanogen bromide fragment.
30   A portion of the mature region of the alkaline
protease gene can be isolated by constructing a larger,
double stranded DNA probe specific for the B. lentus
alkaline protease using the PCR. An analysis based on the
amino acid sequence for the C-terminal cyanogen bromide
35   fragment from the Savinase protease shows that this
sequence has homology with the amino acid sequence around
the active site serine of Savinase, as well as other
subtilisin type serine proteases. The amplified fragment

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is approximately 650 basepairs (bp) in length, in agreement
with the expected size estimated from sequence data
available for other Bacillus alkaline proteases. The 650
bp PCR fragment represents a portion of a gene encoding an
5   alkaline protease, beginning just after the N-terminus of
the mature protease and continuing into the protein coding
region.

The 650 bp fragment can be used to identify the BLAP
gene in the genomic library. After purification, the 650
10   bp fragment can be labeled for example by nick translation
and used to probe gene banks of B. lentus DSM 5483 DNA
which may be constructed by using the cosmid pCP13 and EMBL
lambda vectors. Several cosmid clones carrying DNA with
homology to the 650 bp PCR fragment and to selective
15   oligonucleotide probes may then be identified. Plasmid DNA
of one of these cosmids, pHSH44, has been purified and
characterized by restriction enzyme analysis. Results from
Southern analysis show that a B. lentus DSM 5483 HindIII
fragment of approximately 3.4 kb can hybridize to the
20   radioactively labeled probes 650 bp PCR fragment. The
hybridization, restriction analyses and DNA sequence
(Figure 2) show that the 3.4 kb HindIII fragment having
homology to the 650 bp PCR fragment encodes the BLAP gene.

The fermentation of ATCC 53926 containing an
25   expression cassette which is based on gene fusions
comprising parts of the ATCC 53926 alkaline protease gene
and parts of the BLAP gene is used in the preferred method
of making BLAP on a large scale production basis. Such a
cassette consists of a regulatory region from the ATCC
30   53926 alkaline protease gene comprised of a promoter,
ribosome binding site and initiation codon which is
responsible for the efficient transcription and translation
of the corresponding gene. The regulatory region is
followed by a DNA sequence coding for the signal (pre)
35   peptide, followed by sequences encoding the pro-peptide and
the mature protease. After transcription of the DNA
sequence downstream from the promoter, the messenger-RNA

(m-RNA) is translated into the pre-pro-mature protease, which consists of the signal peptide, the pro peptide and the mature protease. The signal sequence directs the immature protease through the cytoplasmic membrane, where

5   the signal peptide is cleaved off enzymatically by a signal peptidase. The pro sequence seems to insure correct folding and processing of the pro-protease during or after translocation across the cytoplasmic membrane and the cell wall, releasing the mature protease (Power, S. D. et al.

10 (1986) PNAS(USA), 83, 3096; Ikemura, H. et al. (1987) J. Biol. Chem. 262, 7859; Ikemura, H. et al. (1988) J. Biol. Chem. 263, 12959).

Two fusion types identified by the restriction enzyme sites used to join different portions of the ATCC 53926

15 alkaline protease gene and BLAP genes together can be used to prepare the previously described expression cassettes which when used to transform ATCC 53926 will produce BLAP enzyme on a commercial scale. These cassettes are made from ClaI and NcoI fusions.

20   The ClaI fusion combines the ATCC 53926 alkaline protease gene promoter and pre sequence with a DNA fragment encoding the pro and mature sequence of BLAP. A ClaI restriction site can be introduced into the ATCC 53926 alkaline protease gene several codons upstream from the

25 junction of the ATCC 53926 alkaline protease pre and pro sequence. The BLAP gene contains a naturally occurring ClaI site at this position. The promoter and most of the pre region of the ATCC 53926 alkaline protease gene (AvaI/ClaI DNA fragment) can be fused to the (ClaI/SstI)

30 fragment of the BLAP gene which contains a portion of the pre region and all of the pro and mature BLAP sequence. The NcoI and ClaI fusions can be made in E. coli and subcloned into two different Bacillus vectors on an AvaI/SstI DNA fragment.

35   The NcoI fusion produces a mature protease slightly different from the BLAP. The protein made from the NcoI fusion contains threonine at position 3 from the N-terminus

of the mature protease while the BLAP contains serine at the same position (Figure 29). The NcoI fusion can be constructed by combining the promoter, pre and pro sequences of the ATCC 53926 alkaline protease gene with the mature sequence of the BLAP gene. A NcoI restriction site can be introduced into the ATCC 53926 alkaline protease gene several codons downstream from the junction of the pro and mature sequence. A naturally occurring NcoI site is present at a similar position in the BLAP gene. Then, the promoter, pre, pro and four codons of the mature ATCC 53926 sequence (AvaI/NcoI DNA fragment) can be fused to a mature BLAP sequence missing the first four codons (NcoI/SstI DNA fragment). The NcoI and ClaI fusions can be made in E. coli and subcloned into two different Bacillus vectors on an AvaI/SstI DNA fragment.

To further increase enzyme production the ClaI and NcoI fusions can be cloned either with a ATCC 53926 alkaline protease transcription terminator cassette which is a 164 bp DNA fragment, or with a 608 bp C-terminal fragment from the ATCC 53926 alkaline protease gene which is discussed in more detail below.

The ATCC 53926 alkaline protease-BLAP fusion genes can be constructed in the E. coli vector pGM15 (Figure 3), a pTZ19R derivative that contains the 3.4 kb HindIII/KpnI fragment containing the BLAP gene, and then subcloned into Bacillus vectors pBC16 and pUB110. Bacillus fusion gene vector pCB1N (Figure 5) is constructed as shown in Figure 11. Bacillus fusion gene vector pCB11C (Figure 4) can be constructed as shown in Figure 12. Variations of each of the gene fusions described above can be prepared in order to: (a) provide more effective expression of the BLAP protease gene, and (b) to ensure suppression of the expression of the chromosomally encoded ATCC 53926 alkaline protease gene. In order to provide effective termination of the transcription of the BLAP protease gene, the ATCC 53926 alkaline protease transcription terminator can be cloned as a 164 bp DNA fragment downstream of the BLAP

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terminator in each of the gene fusions described above.
Constructs carrying the ATCC 53926 alkaline protease
transcription terminator cassette in the same orientation
relative to the transcription of the ATCC 53926 alkaline
5   protease-BLAP fusion gene in plasmids pCB11C and pCB1N are
designated pCB13C and pCB2N (Figure 6 and 7) respectively.
In order to suppress the expression of the chromosomally
encoded ATCC 53926 protease gene, two additional _Bacillus_
BLAP fusion gene vectors containing a C-terminal fragment
10 of the ATCC 53926 alkaline protease gene can be constructed
in plasmids pCB11C and pCB1N by ligating a 608 bp fragment
of the ATCC 53926 alkaline protease gene synthesized by the
PCR using plasmid pC51 as template.   The 608 bp DNA
fragment can be ligated into the single _KpnI_ site present
15 on vectors pCB11C and pCB1N and new constructs containing
the 608 bp ATCC 53926 C-terminal fragment in the same
orientation relative to the direction of transcription of
the ATCC 53926 alkaline protease-BLAP fusion genes in
pCB11C and pCB1N are designated pCB15C and pCB4N
20 respectively. Restriction maps of these plasmids are given
in Figures 9 and 10 respectively.

     The six ATCC 53926 alkaline protease-BLAP fusion genes
described above and listed below can be subcloned into
Bacillus vectors and transformed into B. _subtilis_ DB104.
25 The ATCC 53926 alkaline protease-BLAP fusion genes can be
subcloned as _AvaI/SstI_ DNA fragments into pBC16 and pUB110
derivatives by digesting the _E. coli_ vectors containing the
ATCC 53926 alkaline protease-BLAP fusion genes (pCB1N,
pCB11C, pCB2N, pCB13C, pCB4N and pCB15C) with _AvaI_ and
30 _SstI_, and ligating them to _Bacillus_ plasmids pC51 and pH70
which have been restricted with _AvaI_, _SstI_, _PstI_ and _SalI_.
Plasmids pC51 and pH70 were cut with _AvaI/SstI_ to provide
the large _AvaI/SstI_ vector fragment containing the
necessary origin of replication and an antibiotic
35 resistance gene (tetracycline resistance in pC51, kanamycin
resistance in pH70) for selection. _SalI_ and _PstI_ can be
used to restrict the small _AvaI/SstI_ fragment containing

the entire ATCC 53926 alkaline protease gene present on
pC51, and pH70 into three smaller DNA fragments. This
decreases the probability of religating the ATCC 53926
alkaline protease gene into the <u>Bacillus</u> vectors.

5   A total of ten ATCC 53926 alkaline protease-BLAP gene
fusion plasmids constructed as described above derived from
the <u>ClaI</u> and   <u>NcoI</u> fusions can be realized in ATCC 53926.
Five <u>Bacillus</u> plasmid constructs derived from the six <u>ClaI</u>
fusions can be transformed into the ATCC 53926 strain.

10   These <u>Bacillus</u> plasmids are pCB55C, pCB56C, pCB58C, pCB75C,
and pCB78C (Figures 13, 14, 15, 16, and 17 respectively).
Five <u>Bacillus</u> plasmid constructs derived from the six <u>NcoI</u>
fusions can be transformed into the ATCC 53926 strain.
These <u>Bacillus</u> plasmids are pCB50N, pCB51N, pCB53N, pCB70N,

15   and pCB73N (Figures 18, 19, 20, 21, and 22 respectively).
        For production of the greatest protease yields, the
ATCC 53926 strain carrying the <u>ClaI</u> fusion constructs
(Figure 23) inserted into a pC51 derivative is preferred.
Construct pCB58C (Figure 15) in the ATCC 53926 strain is

20   the most preferred embodiment for producing the greatest
protease yields.
        The fermentations can be carried out in complex
nutrient media generally known to those skilled in the art.
These media can contain such carbon sources as glucose,

25   sucrose, and/or corn starch. The media can also contain
such nitrogen sources as soy bean meal, cottonseed meal,
casein and potatoe extract. Other growth promoting
ingedients include brewing residue, and corn steep liquor
and inorganic materials such as inorganic phosphates. The

30   media can also contain anti-foams.
        A comparison of the relative protease yields of the
different ATCC 53926 alkaline protease-BLAP constructs in
the ATCC 53926 production strain is shown in Figure 28.
These fermentations were performed with antibiotic

35   selection, using a complex production medium containing
0.5% nitrogen. Protease yields of the ATCC 53926 alkaline
protease-BLAP constructs are presented relative to ATCC

53926 containing plasmid pAM2.3. This plasmid contains the native BLAP promoter and structural gene on an NruI/SalI restriction fragment inserted into plasmid pC51 between the StuI/SalI sites.

5   The fermentation broths obtained by cultivating the different ATCC 53926 alkaline protease-BLAP constructs in the ATCC 53926 production strain typically contain other proteolytic enzymes. However, the BLAP protease in the fermentation broths obtained by fermentation of the

10   different ATCC 53926 alkaline protease-BLAP constructs in the ATCC 53926 production strain is the major proteolytic enzyme in the broths. These fermentation broths contain proteolytic enzymes not present in broths obtained by cultivating Bacillus lentus DSM 5483. Specifically,

15   fermentation broths obtained by fermentation of the different ATCC 53926 alkaline protease-BLAP constructs in the ATCC 53926 production strain produce the BLAP protease and at least one other proteolytic enzyme produced by the ATCC 53926 strain. Preferably, the BLAP enzyme is obtained

20   as a composition comprising the BLAP enzyme which is a mature alkaline proteolytic enzyme from Bacillus lentus DSM 5483 having the amino acid sequence from about amino acid residue 112 to about amino acid residue 380 substantially as shown in Figure 1 and at least one other proteolytic

25   enzyme from Bacillus licheniformis ATCC 53926.
     The proteases produced by cultivating the ATCC 53926 strain containing the various recombinant plasmids are particularly useful in detergent formulations. They can be formulated with surfactants in accordance with methods

30   known in the art of formulating detergent and cleaning compositions especially laundry detergent compositions. A surfactant is any substance which reduces the surface free energy of water including soaps, nonionic surfactants, anionic surfactants, and cationic surfactants. In the case

35   of laundry detergent formulations, the enzymes can be combined with such suitable surfactants as linear alkyl benzene   sulfonates,   ethoxylated   linear   alcohols,

ethoxylated alkyl sulfate, or sulfated linear alcohol. The formulation can also contain builders, fluorescent brighteners, bleach, and other components normally used in the art.

5   DEPOSIT OF MICROORGANISMS

Living cultures of ATCC 53926 and plasmid pCB58C in ATCC 53926, assigned the ATCC Designation 68032, have been accepted for Deposit under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms

10 for the purposes of patent procedure by the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.   A living culture of B. lentus DSM 5483 has been accepted for deposit in Deutsche Sammlung von Mikroorganismen (DSM), Grisebachstr. 8, 3400 Göttingen,

15 Federal Republic of Germany, under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the purposes of patent procedure.

The following examples will serve to illustrate but not to limit the invention.

20   **Example 1.**
Construction of a B. lentus DSM 5483 Gene Bank
A. Using the Cosmid Cloning Vector pCP13

The cosmid cloning vector pCP13 was chosen to construct a genomic library from B. lentus DSM 5483 DNA.

25 This broad host range vector derived from the plasmid pLAFR1 (Friedman et. al. Gene(1982) 18, 289) was obtained from F. Ausubel, Department of Molecular Biology, Massachusetts General Hospital, Boston, MA.   Plasmid pCP13 is 23 kb in size and encodes both tetracycline (Tc^R) and

30 kanamycin (Km^R) resistance, contains multiple unique endonuclease restriction sites, and is mobilizable but not self-transmissible.   Cloning into the BamHI site on pCP13 leads to insertional inactivation of the Km resistance determinant, allowing recombinants to be identified by

35 their Tc^R, Km^S phenotype.   The vector also contains the lambda cos sites which allows concatemeric forms of the cosmid to be packaged into lambda phage particles using

commercially available packaging systems. Inserts in the size range of 12 to 30kb can be cloned into pCP13, making this cloning vector very attractive for the construction of gene banks. Purified pCP13 DNA (20μg) was digested with

5   BamHI (3 units (U)/μg) and subsequently dephosphorylated to prevent recircularization. Purified chromosomal DNA (410 μg) from B. lentus strain DSM 5483 was partially digested with BclI (0.0625U/μg DNA) at 50°C for 60 minutes, and then ligated to the BamHI digested pCP13 DNA. The ligation

10   reaction contained 150 μg/ml pCP13 vector, and 50 μg/ml DSM 5483 chromosomal DNA in a final volume of 30 μl. Ligations were held for 9 hours at 14°C. The ligation mixtures containing concatemeric pCP13-B. lentus genomic DNA fragments were packaged into lambda phage heads using

15   the Packagene Lambda DNA packaging System purchased from Promega, Madison, Wisconsin. The recombinant cosmid molecules packaged into phage particles were used to infect E. coli HB101. E. coli HB101 was grown overnight at 30°C in a rotary shaker at 175rpm in 10 ml of Tryptone Broth or

20   TB (per/liter (1), 10 g tryptone, 5 g NaCl, 2 g maltose and 2.46 g Mg_2SO_4, sterilized by autoclaving). The cells were pelleted and resuspended in 10 mM Mg_2SO_4. Packaging mixture and cells (50μl and 100μl respectively) were mixed together and incubated at 37°C for 20 minutes. Then 1 ml of Luria

25   Broth (per/1, 10 g Bactotryptone, 10 g NaCl, 5 g Yeast extract) was added, and the tubes incubated at 37°C for 60 minutes in New Brunswick G24 rotary shaker at 150rpm. The infected HB101 cells were pelleted by centrifugation (13000 x g) for 5 min at room temperature, resuspended in Luria

30   Broth, and plated onto Luria agar containing 15μg/ml Tc. Fourteen hundred Tc^R Km^S HB101 recombinant clones were obtained. To determine the average insert size, plasmid DNA from 14 randomly selected potential clones was isolated, digested with EcoRI, and analyzed by agarose gel

35   electrophoresis. Eleven of the fourteen clones contained inserts with an average insert size of 25.9kb (range 21.7 to 32.7kb). Based on this large average insert size, and

the assumption that the B. lentus genome is approximately the same size as the E. coli genome, theoretical calculations predicted a 99% probability of detecting the Savinase-like gene by screening 768 cosmid clones.

5   **B. Using the Phage Cloning Vectors EMBL3 and EMBL4**

Another method of cloning large pieces of genomic DNA (>10kb), is to use bacteriophage lambda cloning vectors. These insertion vectors can accept between 9kb and 23kb of heterologous DNA. The heterologous DNA and the lambda DNA
10 are necessary to package the recombinant DNA into phage heads which can then be propagated in an E. coli host. The EMBL3 and EMBL4 phage vectors were purchased from Stratagene in La Jolla, CA. These cloning vectors have been constructed with a polylinker region flanking the
15 internal stuffer DNA which is not needed for replication. When the lambda DNA is restricted with the appropriate enzyme, the remaining two DNA fragments called the left and the right arms needed for replication, can be ligated to heterologous DNA. In order for the recombinant DNA to be
20 efficiently packaged into the phage heads, the ligations must be performed at high DNA concentrations to favor the formation of concatamers, and the heterologous insert must be between 9kb and 23kb in size. This latter requirement is based on the observation that the length of the DNA
25 which can be packaged is between 70% and 105% of the size of the wild-type lambda genome. Further, both EMBL systems take advantage of spi selection (sensitive to bacteriophage P2 inhibition). EMBL arms ligated to the stuffer fragment and packaged into phage heads cannot grow on a host strain
30 containg a P2 lysogen. In this system, it is unlikely that stuffer fragment and EMBL arms do religate. If this occurs, these phage will not replicate when plated on the appropriate E. coli host.

In order to prepare genomic libraries of DSM 5483 DNA
35 in either the EMBL3 or EMBL4 vectors, DSM 5483 DNA was partially digested with either EcoRI or BclI (the 5' sticky end produced by BclI is complementary to the ends of the

vector cut with <u>Bam</u>HI). In each case, the genomic DNA was digested with less than 1 unit of enzyme per microgram of DNA for 60 minutes. Optimal packaging efficiencies are obtained with lambda DNA's that are concatemeric.

5 Therefore, the ligation reactions were set to achieve DNA concentrations of 200μg/ml, which favors concatemer formation. In a standard 5μl ligation reaction, 1μg of vector arms was mixed with 200 to 400 ng of genomic DNA. Ligation for 1 hour at room temperature was followed by an

10 overnight incubation at 4°C. Concatemeric DNA from the ligation reactions was packaged into phage heads using GigaPack Plus <u>in</u> <u>vitro</u> packaging extract purchased from Stratagene (La Jolla, California), and the protocol provided with the extracts. After packaging, 0.5ml of

15 phage dilution buffer was added to the packaging reactions (per liter, 5.0 g NaCl, 2 g MgSO$_4$·7H$_2$O, 50ml 1M Tris HCl pH7.5 and 5ml 2% gelatin) and 20μl of chloroform. The packaging reactions were titered, and amplified according to the protocols provided by Stratagene. From each phage

20 library 5 X 10^5 recombinant phage were obtained. When a similar experiment was performed with a test insert provided with the commercial cloning kit, 3 X 10^6 recombinant phage were obtained. Less than 5% of the plaques generated by the control insert were due to

25 background phage. Recombinant phage had an average insert size of 12-14kb with about 70% of the phage actually showing inserts. The phage libraries were amplified to a titer of 10^{8}phage/ml and stored over chloroform at 4°C. Other stocks were prepared for longer term storage at -

30 70°C.

To characterize the EMBL libraries and to find the Savinase-like gene, the following approach was undertaken. Groups of plaques were isolated from either the EMBL3 or the EMBL4 libraries, and their DNA was isolated by a phage

35 minipreparation procedure (Maniatis <u>et</u>. <u>al</u>., (1982), A Laboratory Manual, Cold Spring Harbor Laboratories, Cold Spring Harbor, New York). The insert genomic DNA present

in the EMBL3 and EMBL4 libraries was digested with either
SalI or _EcoRI_, respectively, and after agarose gel
electrophoresis the DNA fragments were transferred to Gene
Screen Plus. Total DSM 5483 genomic DNA was nick translated
5   and used as a hybridization probe to verify that the
inserts present in the EMBL libraries were really DSM 5483
DNA. As expected, the cloned inserts hybridized with the
labelled DSM 5483 DNA. In a second experiment, genomic DSM
5483 DNA was cut with either _EcoRI_ or _BclI_, electrophoresed
10 in an agarose gel, and then transferred to Gene Screen.
DNA isolated from groups of recombinant phage from either
the EMBL3 or EMBL4 libraries was nick translated and used
as a hybridization probe. As expected, hybridization to
DSM 5483 genomic DNA occurred while little or no
15 hybridization was detected to DNA from other _Bacillus_
species. These results verified the formation of gene
libraries of _B. lentus_ DSM 5483 chromosomal DNA using the
lambda EMBL cloning vectors.

Example 2.

20 **Construction of DNA Probes Specific for the Savinase-like
Protease Gene Present in B. lentus DSM 5483**

    A. Oligonucleotide Probes

    The gene bank of DSM 5483 chromosomal DNA constructed
in _E. coli_ with the cosmid pCP13 was screened using
25 oligonucleotide probes. A series of probes of varying
degeneracy were synthesized using a Biosearch DNA
synthesizer. The sequence of these oligonucleotides was
deduced from amino acid sequence data obtained from the N-
terminus of the Savinase protease and from the C-terminal
30 fragment of Savinase derived by cyanogen bromide cleavage.
The sequence and location of these probes in relation to
the Savinase protease are depicted in Figures 24 and 25.
The NO2 and NO4 series of probes were both designed from N-
terminal amino acid sequence data. The NO2 series was 23
35 bases in length with 14 bases of homology and contained 16
fold degeneracy, while the NO4 series were 17 bases in
length with 48 fold degeneracy. The NO4.1-4 series of

probes was designed using the most probable codon usage for
B. subtilis. The CO3 series of oligonucleotides were all
17 bases in length and had a 32 fold degeneracy. These
latter probes were designed from amino acid sequence
derived from the C-terminal cyanogen bromide fragment of
Savinase. These probes were purified, radioactively
labeled, and hybridized to chromosomal DNA from DSM 5483
which had been restricted, electrophoresed on agarose gels,
and blotted onto nitrocellulose using the procedure of
Southern. The restriction enzymes used included EcoRI,
HindIII, TaqI, Sau3A, SstI, XbaI, and XhoI. The strongest
hybridization signals were obtained with oligonucleotides
NO2.2, NO4.2, and CO3.3 (Figure 25). The latter three
labeled oligonucleotides were used to screen Southern blots
of restricted chromosomal DNA's from E. coli HB101, B.
subtilis DB104, B. licheniformis, and the B. lentus strain,
and were found to hybridize only to DNA fragments from the
B. lentus strain. This result confirmed that these three
oligonucleotides were specific for DNA encoding the
Savinase-like protease.

**B. Amplification of a Portion of the Alkaline Protease
Gene Sequence**

        Although three of the oligonucleotide probes described
above were specific for DSM 5483 chromosomal DNA an
additional approach was pursued in order to isolate a
larger, double stranded DNA probe specific for the Savinase
-like alkaline protease. The N-terminal and internal
oligonucleotides described above were utilized as primers
in the PCR to amplify that region of DSM 5483 chromosomal
DNA extending from the N-terminus to amino acid residue 222
of the mature alkaline protease (Figure 24). The amino
acid sequence obtained from the C-terminal cyanogen bromide
fragment from Savinase had homology with the amino acid
sequence around the active site region of other serine
proteases. If the distance from the N-terminus of Savinase
to the active site region was similar to other serine

proteases, then the expected size of the amplified fragment would be approximately 650 bp.

The PCR was carried out using a commercial GeneAmp kit purchased from Perkin - Elmer Cetus Instruments. In the
5   PCR, the DNA segment to be amplified may range from a few hundred bp to more than 5 kb in length, and is contained on either a plasmid or on a chromosomal DNA template. Amplification requires the synthesis of two oligonucleotide primers, approximately 15 to 25 bases in length, designed
10 to have homology to opposite DNA strands at either end of the DNA segment. Since DNA synthesis occurs in a 5' to 3' direction, each primer is designed to mimic the corresponding 5' to 3' strand. In the initial experiment, 16 different PCR's were performed using all possible
15 combinations of probe series CO3 with NO2, and DSM 5483 chromosomal DNA as template. The concentration of template and primers was as prescribed in the kit. The PCRs were initiated by mixing template, excess primers, dNTP's, heat stable DNA polymerase, and reaction buffer in eppendorf
20 tubes which were cycled for set periods of time at 94°C, 37°C, and 72°C. In each cycle, the double stranded DNA template was denatured by heating at 94°C, then cooled to 37°C to allow for annealing of the primers to the appropriate DNA sequences on the single stranded template.
25 The tubes were then shifted to 72°C, where complementary DNA strands were synthesized through elongation of the annealed primers by a heat stable DNA polymerase (Taq polymerase). This process was repeated 25 times, with the amount of amplified DNA doubling after each cycle. One of
30 the PCR (oligonucleotide pair NO2.2 and CO3.3) resulted in the amplification of a single DNA fragment approximately 650 bp in length. Approximately 3 μg of the 650 bp fragment was generated by the PCR, representing about a 5 x 10^4 fold amplification. The PCR was repeated using all
35 combinations of the NO4 and CO3 series of probes. Based on the previous result, the combination of NO4.2 and CO3.3

would be expected to produce the amplified 650 bp fragment, and in fact this was the case.

Example 3.

Identification of Cosmid Clones Carrying the Savinase-like
5   Alkaline Protease Gene From B. lentus

To screen for the Savinase-like gene, the 1400 Tc^R Km^S HB101 clones were divided into 13 pools of 100-150 clones each. Plasmid DNA from each pool, and from a culture containing the entire gene bank was purified and digested
10   with EcoRI. Restriction fragments were separated by agarose gel electrophoresis and transferred to Gene Screen Plus hybridization membrane (DuPont, Wilmington, Delaware) by the methods provided by the manufacturer. In order to detect the Savinase-like gene within the cosmid bank,
15   oligonucleotide probes were synthesized. The predicted sequence of the probes was based on previously acquired amino acid sequence data for the Savinase protease. These probes also served as primers for the synthesis of a 650 bp portion of the BLAP gene using the PCR (described above).
20   The 650 bp PCR fragment was labelled with ^{32}P in a nick translation reaction (protocol obtained from NEN, Wilmington, Delaware) and used as a hybridization probe. This fragment was shown to contain an internal EcoRI site. Therefore, two bands of hybridization would be detected
25   when a complete B. lentus Savinase - like protease gene was present within a cosmid pool digested by EcoRI. When cosmid DNA isolated from the total gene bank of DSM 5483 DNA was digested with EcoRI, two fragments of 6.35 and 2.64 kb were found to hybridize with the labeled 650 bp PCR
30   probe. In six of the recombinant cosmid pools (#3,#4,#8,#11,#12 and #13), these same bands of hybridization were detected, indicating that the Savinase-like gene was present in at least one cosmid clone contained within each of these six pools. Pool #13, which
35   possessed 150 E. coli transformants, was subdivided into 12 subpools of 12 or 13 transformants each. Plasmid DNA from each subpool was isolated, digested with EcoRI, and the DNA

fragments separated by gel electrophoresis. After transfer
to a Gene Screen Plus membrane, the filter was hybridized
to the labeled 650bp PCR fragment. Two subpools (#7 and #9)
were found to contain the same bands of hybridization as

5   detected initially in Pool #13. Plasmid DNA was isolated
from each individual transformant in subpools #7 and #9 and
the plasmid DNA's were digested with either EcoRI or
HindIII in separate reactions. After agarose gel
electrophoresis and transfer to Gene Screen Plus, the

10 hybridization procedure was repeated. Cosmid DNA from
subpool #9 clone #5 exhibited the same pattern of
hybridization expected of a clone which contained the
Savinase-like protease gene. When cosmid DNA from clone #5
and DSM 5483 chromosomal DNA were digested with HindIII and

15 probed with the 650bp PCR fragment, the same band of
hybridization was detected for both. A fragment size of
3.4 kb was calculated from the location of the
hybridization band in relation to known molecular weight
standards. The cosmid from subpool #9 clone #5 was

20 designated pHSH44.

    A large scale preparation of the cosmid pHSH44 was
prepared by CsCl- ethidium bromide gradient centrifugation,
and after dialysis, restricted with HindIII. The HindIII
fragments were separated by agarose gel electrophoresis,

25 and the 3.4 kb HindIII fragment which hybridized with the
650bp PCR fragment was purified, and subcloned into the
commonly used E. coli vectors pUC19 and pBR322 for
restriction analysis and further subcloning into Bacillus
vectors.

30   **Example 4**
**Genetic Characterization of the B. lentus Alkaline Protease
Gene A. Subcloning and Characterization of a Restriction
Fragment Carrying the B. lentus Alkaline Protease Gene**

    After purification, the 650bp fragment was labeled by

35 nick translation and used to probe gene banks of DSM 5483
DNA constructed using the cosmid pCP13 and EMBL lambda
vectors. In both cases, using very stringent hybridization

conditions positive clones were identified. From
hybridization data, several cosmid clones carrying DNA with
homology to the 650 bp PCR fragment and selective
oligonucleotide probes were identified. Plasmid DNA of one
5 of these cosmids, pHSH44, was purified and characterized by
restriction analysis. Results from Southern blots
indicated that a DSM 5483 HindIII fragment of approximately
3 to 5 kb hybridized to radioactive probes prepared from
the 650 bp PCR fragment. Cosmid pHSH44 carried three
10 HindIII restriction fragments of 3.4 kb, 4.0 kb, and 4.8
kb. Initially, all of these fragments were purified by
agarose gel electrophoresis and cloned into both pUC19 and
pBR322. The ligation reaction in all cases contained 100
ng of dephosphorylated vector DNA and approximately 1000 ng
15 of the corresponding HindIII fragment in a volume of 20 µl.
These ligation mixtures were transformed into competent
cells of either E. coli DH5∝ (for pUC19), or E. coli HB101
(for pBR322). Ampicillin resistant transformants were
selected and screened for the correct plasmid constructs.
20 All of the HindIII fragments were cloned into both vectors.
The correct constructs in pUC19 and pBR322 were designated
pMG102.2, and pMG105.9, respectively. The results from
hybridization and restriction analysis identified the 3.4
kb HindIII fragment (present in plasmids pMG102.2 and
25 pMG105.9) as having homology to the 650 bp PCR fragment.
Plasmid pMG102.2 was purified, and characterized by
restriction analysis with 26 different enzymes. In order
to obtain the complete nucleotide sequence of the 3.4 kb
HindIII fragment, it was necessary to subclone portions of
30 the fragment into the pTZ sequencing vectors. All
subclones were made using DNA fragments of pMG102.2
purified from low melting temperature agarose after gel
electrophoresis. The excised DNA fragments were ligated to
vector DNA which had been restricted at the appropriate
35 sites, and transformed into E. coli DH5∝ cells. Ampicillin
resistant transformants were selected and the correct
clones identified by restriction analysis. Plasmid DNA's

from the correct constructs were purified from the DH5∝
strain and transformed into E. coli NM522 to provided the
single stranded templates necessary for DNA sequencing.

    B. Sequencing of the 3.4 kb HindIII Fragment
5 **Containing the Alkaline Protease Gene of B. lentus**

    The 650 bp PCR fragment was also cloned into E. coli
plasmid pUC19. The PCR fragment was blunt-end ligated to
pUC19 previously digested with SmaI, and transformed into
E. coli DH5∝ purchased from Bethesda Research Labs (BRL),
10 using the procedure provided by BRL. Transformants were
selected on X-gal plates containing 50 μg/ml ampicillin
(Maniatis, et al., ibid). Cloning of the 650 bp fragment
into the SmaI site would be expected to disrupt lacZ
complementation, and therefore produce white colonies on
15 the X-gal plates. Approximately 200 of 10000 colonies on
the X-gal plates were white. Plasmid DNA was prepared from
12 of the 200 transformants and analyzed for the presence
of the 650 bp fragment. Seven of the 12 had the PCR
fragment cloned into pUC19 in the same orientation. This
20 plasmid was designated pMG100.13. For DNA sequencing, the
650bp fragment was removed from pMG100.13 by double
digestion with BamHI and KpnI. Recognition sites for these
enzymes are contained in the polylinker sequence originally
from pUC19 but are not found within the DNA sequence of the
25 650bp PCR fragment. The BamHI/KpnI PCR fragment was
ligated with the commercial (Pharmacia) sequencing vectors
pTZ18R and pTZ19R, digested with the same enzymes, and
transformed into E. coli. Transformants containing the
correct constructs were identified by isolation of plasmid
30 DNA and restriction analysis. The two new constructs
represented both orientations of the PCR fragment in
relation to a sequencing primer site located on the pTZ
vectors. DNA sequencing was performed using a dideoxy
procedure and a commercially available sequencing primer
35 from the M-13 system. The initial sequencing data
consisted of approximately 220 bases for each strand,
beginning at either end and extending internal into the 650

bp PCR fragment. This initial sequence confirmed that: the 650bp PCR fragment had been cloned into the SmaI site of pUC19 as a blunt end fragment; the DNA sequence immediately following the SmaI site in the pTZ18 clone is

5   consistent with the NO2.2 primer; the 17 bp of DNA sequence immediately following the SmaI site in the pTZ19 clone (opposite orientation to pTZ18) is in agreement with the CO3.3 primer; and the amino acid sequences deduced from the DNA sequences both within and extending out from

10 the NO2.2 and CO3.3 primer regions were in agreement with the N-terminal and internal amino acid sequences determined from Savinase. Based on the above results, it can be concluded that the 650bp PCR fragment amplified from B. lentus DSM 5483 DNA represents a portion of a gene encoding

15 an alkaline protease, beginning just after the N-terminus of the mature protease and continuing into the protein coding region. To complete the sequencing of the 650 bp PCR fragment, two approaches were pursued. First, the pTZ19R vector carrying the 650 bp PCR fragment was digested

20 with EcoRI to remove a portion of the PCR fragment, religated, and transformed into E. coli. Preliminary restriction analysis of the PCR fragment had revealed an internal EcoRI site, with a second EcoRI site present in the polylinker region of the vector. The correct subclone

25 isolated from this experiment contained the EcoRI site adjacent to the priming site for DNA sequencing, allowing the determination of additional internal DNA sequence of the PCR fragment. At the same time, the small EcoRI fragment removed from the pTZ19R PCR construct was cloned

30 in the opposite orientation into pTZ18R allowing the DNA sequence in the opposite direction from the EcoRI site to be determined.

     C. Determining the Transcriptional Start Site for the
        B. lentus 5483 Alkaline Protease Gene

35   A necessary step in the genetic manipulation of the cloned BLAP gene is the determination of the start site of transcription. A primer extension protocol was used to

identify this site (Arnosti and Chamberlain (1989),
PNAS(USA), 86, 830). In this procedure, a primer (20 to 30
bases in length) was synthesized which had homology to a
sequence on the template DNA positioned 200 to 300 bp 3' to
5   the suspected start of transcription. The primer was
hybridized with the messenger RNA transcript under
stringent conditions, and then the primer was elongated
using the enzyme reverse transcriptase. The size of the
reverse transcript was measured in a denaturing
10   polyacrylamide gel. The elongated primer was also
sequenced so that the exact base of transcription
initiation could be read off of the sequencing gel. Total
RNA was isolated as described by Arnosti and Chamberlin
(ibid) from DSM 5483 or from the B. subtilis strain DB104
15   containing plasmid pJW5. Plasmid pJW5 was formed by cloning
the 3.4 kb BLAP HindIII fragment into the HindIII site of
plasmid pC194. Lysozyme treatment followed by sonication
was used to disrupt Bacillus cells. To purify the RNA from
contaminating nucleases, at least 4 to 5 phenol extractions
20   were performed. The size of the BLAP gene transcript was
determined by Northern analysis, using the protocol
described by Thomas ((1983), Methods in Enzymology 100,
255). In this experiment, total Bacillus RNA was denatured
and then analyzed by electrophoresis through an agarose gel
25   containing a denaturing concentration of formaldehyde (BRL
Focus 9:3,14). The RNA was then transferred from the gel
to a nitrocellulose filter which was then baked at 60°C for
2 hours. Plasmid pMG109.3 (pUC19 + the 3.4 kb HindIII
fragment containing the BLAP gene) was nick-translated and
30   used to probe the Northern blot. The transcript produced
by the BLAP gene was approximately 1200 to 1400 bases in
length. Two oligonucleotide primers were designed on the
basis of the BLAP nucleotide sequence. These primers were
homologous to BLAP sequences between the NcoI and the HpaI
35   sites, and 5' to of the sequence encoding the mature
protease peptide. Primer A spans the BLAP nucleotide
sequence included in Figure 2 between bases 2056-2033, and

primer B is found between bases 2242-2222. The results of
the primer extension experiments using either primer and
messenger RNA prepared from either B. lentus DSM 5483, or
B. subtilis DB104 carrying plasmid pJW5 was the same. In
5   both cases, the start site of transcription was determined
to be an adenine residue at position 1924 in the BLAP
sequence. This adenine is located 42 bp upstream of the
ATG translation initiation codon at the beginning of the
BLAP signal peptide. On the basis of this result, the
10   length of the BLAP message is 1210 bases.

Example 5.

**Construction of Fusions Between the B. licheniformis ATCC
53926 Alkaline Protease Gene and the B. lentus Alkaline
Protease Gene (BLAP)**

15   **A. Experimental Approach**

For large-scale production of BLAP, an expression
system was designed which was based on gene fusions
comprising parts of the ATCC 53926 alkaline protease gene
and parts of the BLAP gene. The native promoter of the
20   BLAP gene proved to be insufficient for the effective
production of BLAP in B. subtilis DB104 as well as DSM
5483. It was known from fermentation results that the
promoter of the ATCC 53926 alkaline protease gene worked
well for the large scale production of Carlsberg-type
25   protease in ATCC 53926. Thus, a fusion of the promoter of
the ATCC 53926 alkaline protease gene to the structural
gene of BLAP potentially would provide an efficient
expression system for protease similar to commercially
available serine proteases in ATCC 53926. Genes coding for
30   alkaline proteases such as ATCC 53926 and BLAP typically
encode a pre-pro form of the enzyme. They all start with
a regulatory region comprised of a promoter, ribosome
binding site and initiation codon, which is responsible
for the efficient transcription and translation of the
35   corresponding gene. The regulatory region is followed by
a DNA sequence coding for the signal peptide. The signal
sequence which is required for the secretion of the

protease outside the cell is followed by a coding region for pro-peptide and the mature protease. After transcription of the DNA sequence downstream from the promoter, the messenger RNA (mRNA) is translated into the

5   final gene product. The pre-pro-mature protease consists of the signal peptide, the pro peptide and the mature protease. The signal sequence directs the immature protease through the cytoplasmic membrane, where the signal peptide is cleaved off enzymatically by a signal peptidase.

10 The pro sequence seems to be essential for a correct folding and processing of the pro-protease during or after translocation across the cytoplasmic membrane and the cell wall. Amino acid sequence comparison between the BLAP and ATCC 53926 alkaline protease gene revealed 49% homology in

15 the pre region, 38% homology in the pro region and 75% homology in the mature region. Because the interaction between the pre and pro regions necessary to obtain correct secretion and maturation of a protease is not fully understood, two different fusions between portions of the

20 ATCC 53926 alkaline protease gene and portions of the BLAP gene were constructed. The two basic fusions were identified by the restriction enzyme sites used to join different portions of the ATCC 53926 and BLAP genes together.

25   **1. ClaI Fusion**

In order to construct the ATCC 53926 alkaline protease-BLAP ClaI gene fusion, an extra ClaI restriction site had to be introduced into the ATCC 53926 alkaline protease gene at position 376 next to the junction of the

30 ATCC 53926 pre and pro sequence (Figures 27 and 27a). At basepair 2032 the BLAP gene already contained a naturally occurring ClaI site at the corresponding position. The ClaI site was introduced into the ATCC 53926 alkaline protease gene by using the PCR. Plasmid pGM15 described

35 previously was isolated from NM522, a dam$^+$ E. coli strain and restricted with AvaI, ClaI and MluI yielding four DNA fragments of 4330 bp, 1296 bp, 382 bp and 147 bp. The ClaI

sites at positions 1206 and 2032 in the BLAP sequence
(Figure 2) were not restricted due to dam-methylation at
overlapping GATC sequences. Restriction with MluI was used
in order to generate three DNA fragments out of the 1825 bp

5   AvaI/ClaI fragment and to prevent religation and recloning
of these fragments to the 4330 bp AvaI/ClaI fragment. The
DNA fragments from the pGM15 AvaI/ClaI/MluI digest were
ligated to a DNA fragment containing the ATCC 53926
alkaline protease promoter and pre sequence, which was

10 synthesized using the PCR. This 292 bp fragment was
flanked by AvaI and ClaI restriction sites at its 5' and 3'
ends, respectively, and was synthesized using plasmid pC51
as template with primers #1 and #3 (Figure 8). The PCR
fragment contained sequence information from the N-terminal

15 part of the ATCC 53926 alkaline protease gene including the
ATCC 53926 alkaline protease promoter, and most of the ATCC
53926 pre sequence (from the AvaI site at basepair 84 and
extending to the newly formed ClaI site at basepair 376).
The above ligation was transformed into E. coli NM522, and

20 ampicillin-resistant transformants were selected. Plasmid
DNA's were prepared and characterized by restriction
analysis.   A   correct   construct   was   identified   and
designated pCB10C (Figure 12). Plasmid pCB10C contained
the ATCC 53926 alkaline protease promoter and most of the

25 ATCC 53926 pre sequence fused to the BLAP sequence
downstream from the native ClaI site at position 2941.
Plasmid pCB10C was used to generate the final ClaI-fusion
gene by linearizing it with ClaI and inserting a 909 bp
ClaI fragment from the BLAP gene which contained the

30 sequence between the ClaI sites at position 2032, and
position 2941. The 909 bp ClaI fragment was generated by
hydrolyzing plasmid pMG108.2 (Figure 26) with ClaI.
Plasmid pMG108.2 was isolated from E. coli GM33 (dam⁻) in
order to prevent dam methylation at adenine residues in the

35 recognition tetranucleotide GATC. The 909 bp DNA fragment
was isolated from a preparative agarose gel and ligated
into plasmid pCB10C previously having been restricted with

<u>Cla</u>I and dephosphorylated with bacterial alkaline phosphatase. The ligation mix was transformed into <u>E. coli</u> NM522. Ampicillin resistant transformants were screened and the correct construct (pCB11C) was identified. A

5 restriction map of pCB11C is given in Figure 4 and a schematic for its construction is given in Figure 12. Analysis of the DNA sequence across the fusion of the ATCC 53926 and BLAP genes present in plasmid pCB11C confirmed the expected DNA and amino acid sequences.

10   **2. NcoI Fusion**

In order to construct the ATCC 53926-BLAP **Nco**I **gene** fusion, an extra <u>Nco</u>I restriction site had to be introduced into the ATCC 53926 alkaline protease gene at position 638 near to the junction of the ATCC 53926 pro and mature

15 sequence (Figures 27 and 27a). The BLAP gene contained a naturally occurring <u>Nco</u>I site at position 2311. The <u>Nco</u>I site was introduced into the ATCC 53926 sequence by using the PCR. A 554 bp PCR DNA fragment was synthesized using plasmid pC51 as template, and oligonucleotides #1 and #2

20 (Figure 8) as primers. The design of the primers introduced <u>Ava</u>I and <u>Nco</u>I sites at the 5' and 3' ends of the fragment, respectively. The 554 bp fragment contained the sequence information for the ATCC 53926 alkaline protease promoter, pre and pro sequences, and the first four amino

25 acids of the mature ATCC 53926 protease gene. After restriction with <u>Ava</u>I/<u>Nco</u>I the PCR fragment was ligated to the 4958 bp <u>Ava</u>I/<u>Nco</u>I DNA fragment from plasmid pGM15. Plasmid pGM15 (Figure 3) was restricted with <u>Ava</u>I, <u>Nco</u>I and <u>Mlu</u>I yielding four DNA fragments of 4958 bp, 668 bp, 382

30 bp, and 147 bp. <u>Mlu</u>I was used to generate three fragments out of the 1197 bp <u>Ava</u>I/<u>Nco</u>I fragment to prevent religation of this fragment to the 4958 bp <u>Ava</u>I/<u>Nco</u>I fragment. The ligation mix was transformed into <u>E. coli</u> NM522. Ampicillin resistant transformants were screened and the

35 correct construct (pCB1N) (Figure 5) identified. Analysis of the DNA sequence across the <u>Nco</u>I site of plasmid pCB1N confirmed the expected DNA and amino acid sequence. The

NcoI fusion alkaline protease gene contained the entire
promoter, pre- and pro sequence of the ATCC 53926 and the
entire sequence of the mature BLAP with a single amino acid
substitution at amino acid residue 3 of the mature BLAP

5   sequence where a threonine replaced a serine.

Example 6.

Subcloning of the B. licheniformis ATCC 53926 - BLAP Fusion Gene into Bacillus Vectors and Transformation into the ATCC 53926 Production Strain

10   After the ATCC 53926 alkaline protease-BLAP fusion
genes had been cloned into E. coli vectors as previously
described, they were subcloned into Bacillus vectors and
transformed into B. subtilis DB104. The ATCC 53926
alkaline protease-BLAP fusion genes were subcloned as

15 AvaI/SstI DNA fragments into pBC16 and pUB110 derivatives
by restricting the E. coli vectors containing the ATCC
53926 alkaline protease-BLAP fusion genes (pCB1N, pCB11C,
pCB2N, pCB13C, pCB4N and pCB15C) with AvaI and SstI, and
ligating them to Bacillus plasmids pC51 and pH70 which had

20 been restricted with AvaI, SstI, PstI and SalI. Plasmids
pC51 and pH70 were cut with AvaI/SstI to provide the large
AvaI/SstI vector fragment containing the necessary origin
of   replication   and   an   antibiotic   resistance   gene
(tetracycline resistance in pC51, kanamycin resistance in

25 pH70) for selection. Restriction with SalI and PstI was
done to hydrolyze the small AvaI/SstI fragment containing
the entire ATCC 53926 alkaline protease gene present on
pC51, and pH70 into three smaller DNA fragments. This
decreased the probability of religating the ATCC 53926

30 alkaline protease gene into the Bacillus vectors. The
initial cloning experiments into Bacillus were done using
B. subtilis DB104 as recipient. Since DB104 is a reduced
protease strain, it provides a direct screen for the
presence and expression of the BLAP gene. Using pH70 and

35 pC51 as recipients of the AvaI/SstI fragment from each of
the E. coli, ATCC 53926 alkaline protease-BLAP fusion
plasmids described above resulted in a total of 12

potential _Bacillus_ plasmid constructs. Figure 23 depicts
the six ATCC 53926-BLAP plasmids derived from the three
ClaI fusions present in _E. coli_ plasmids pCB11C, pCB13C,
and pCB15C. Detailed restriction maps of the plasmids that
5   were transformable into the ATCC 53926 production strain,
plasmids pCB55C, pCB56C, pCB58C, pCB75C, and pCB78C are
shown in Figures 13, 14, 15, 16, and 17 respectively. All
five of these constructs were transformed into _B. subtilis_
DB104 and into the ATCC 53926 production strain. In a
10   similiar fashion, the _NcoI_ ATCC 53926 alkaline protease-
BLAP fusions were cloned from pCB1N, pCB2N, and pCB4N into
pH70 and pC51. Five of the six plasmids from this cloning
(pCB50N, pCB51N, pCB53N, pCB70N, and pCB73N) were
transformed into _B. subtilis_ DB104, and into the ATCC 53926
15   production strain. The restriction maps for these
constructs are shown in Figures 18, 19, 20, 21, and 22
respectively.

<div align="center">

Example 7

Protocol for Production of B. _lentus_ Alkaline Protease
20   **(BLAP) by ATCC 53926 Alkaline Protease-BLAP Strains in**
Shake Flask Cultures.
</div>

To compare derivatives of _B. licheniformis_ ATCC
53926 carrying the different ATCC 53926 alkaline protease-
BLAP plasmids, a three-stage shake flask procedure was
25   used. In these experiments, the antibiotic tetracycline or
kanamycin was always present in the media (described below)
to select for the desired plasmid. The first preculture
flask was inoculated from a frozen stock vial that had been
thawed at room temperature. After 7 hours of incubation at
30   39°C and 200 rpm, 1.0% (v/v) of this culture was transferred
to a fresh (second) preculture flask and the incubation
resumed. Between 13 and 15 hours, a 1% transfer was made
to duplicate main culture flasks. Culture samples were
obtained periodically from the main culture flasks between
35   27 and 50 hours for determination of protease activity.
The shake flask experiments used a complex medium that
contained assimilable carbon and nitrogen sources for the

microorganism as well as other organic materials and inorganic salts. The major components included hydrolyzed corn starch, sodium caseinate, soy meal, a brewing residue, and corn steep liquor. Small amounts of antifoam,
5   inorganic salts and vitamins B1 and B6 were also present. After distribution to baffled erlenmeyer flasks the medium was sterilized by heating to 121°C at 15 psi.

Example 8

Protocol for Small-Scale Fermentation of ATCC 53926
10   **Derivatives that Produce BLAP.**

The fermentations were carried out in two steps where the organism was first grown up in a shake flask containing a complex medium with antibiotic to select for the endogenous plasmid DNA. After achieving a high cell
15   density, part of the flask culture was transferred to the main fermenter vessel for continued growth and the production of protease. The vessels were Biostat E or ES manufactured by B. Braun, Inc.. All fermentations contained less than 10 liters of culture, and were carried
20   out with BL1 (Biosafety Level One) containment precautions and with good laboratory practices as specified in the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (NIH Guidelines, May 7, 1986).

25   The shake flask and fermenter media were very similar complex media with an assimilable carbon source, assimilable nitrogen source, organic nutrients and inorganic salts which allowed vigorous growth of the microorganism as well as production of protease. Major
30   medium components included deionized water, corn starch, soy meal, sodium caseinate, a brewing residue, and corn steep liquor. Minor components were $(NH_4)_2HPO_4$ and Na_2HPO_4. A commercial antifoam was also present in the fermenter media. The medium was sterilized by heating to 121°C at 15
35   psi. The described media in a two-liter baffled flask was inoculated from 1.0 ml of frozen culture-glycerol stock (see stock preparation) that had been thawed at room

temperature. Either tetracycline or kanamycin antibiotic
was added to the shake flask culture to select for the
cells that carried the desired plasmid. The shake flasks
were incubated at 39°C with slow shaking to grow the
5   culture. The fermenter media was eventually inoculated
with a 1.5% (v/v) transfer of the shake flask culture.
Antibiotic was also present in the fermenter media. The
agitation rate and airflow were held at 1,300 rpm and 1 vvm
(liters/min.), respectively. In a few experiments, the
10 agitation and airflow rate had to be increased for a brief
period during log phase growth to maintain a partial oxygen
pressure (pO_2) of greater than 20% in the culture.

During cultivation of the production strain, the
culture pH and temperature were changed at specified times.
15 In particular, it was found that control of the culture pH
had a significant effect on production of protease. The
culture pH was controlled at 6.8 from 0-13.5 hours, then
controlled at pH 7.5 from 18 to 35 hours. Sterile
solutions of KOH and H_2SO_4 were added to the media as needed
20 to control pH. From the start of the fermentation the
culture temperature was 39°C. The temperature was lowered
to 34°C after 14.5 hours of incubation.

Example 9

Assays for the <u>B</u>. <u>lentus</u> Alkaline Protease

25   For assays of samples from shake flasks or
fermentations, the protease was separated from the cells
and media debris by centrifugation. The protease in these
crude preparations was quantified as described below.

The HPE (Henkel Protease Einheit) method was based
30 on a discontinuous assay of the release of acid-soluble
fragments from protease digests of casein. About 50 μl of
protease solution was combined with 750 μl of a casein
solution prepared by heating Hammarsten casein in
tripolyphosphate-tris buffer solution at pH 8.5. After 15
35 minutes of incubation in a 2.2 ml eppendorf tube at 50°C,
600 μl of 0.44M trichloroacetic acid solution was added.
The mixture was chilled on ice for 15 minutes before

removing the precipitate by centrifugation. Acid-soluble
peptides were measured in a spectrophotometer at 290 nm.
Two HPE units were defined as being equal to an absorbance
change of 0.500 OD at 290 nm.

5   The EPE (Esterolytic Protease Einheit) method was
useful primarily for detection of activity from the ATCC
53926 production strain. The kinetic assay was started by
mixing 100 μl of a sample or known standard of a commercial
protease (Maxatase, Novo A/S) into an acetone buffer
10   containing CBZ-valine-p-nitrophenyl ester. The rate at
which p-nitrophenol was released by esterolytic cleavage of
the CBZ ester was monitored spectrophotometrically at A_{340}
and determined by linear regression analysis. Units of EPE
activity for a sample were determined by comparison of the
15   reaction rate of the sample to that of the Maxatase
standard.

Example 10.
Distinguishing between BLAP and B. licheniformis ATCC 53926
Protease by Gel Electrophoresis

20   The B. lentus alkaline protease produced by the ATCC
53926 alkaline protease-BLAP fusion strains could be
distinguished from ATCC 53926's native extracellular
proteolytic enzymes by electrophoresis in homogeneous, non-
denaturing ("native") 12.5% polyacrylamide (PA) gels with
25   pH 6.0 buffer. Crude samples obtained by centrifugation of
the fermenter broth were resolved by this method on the
PhastSystem apparatus manufactured by Pharmacia, Inc.. The
protein species were visualized by staining the PA gel in
Coomassie Blue.

30   ## Example 11
Preparation of B. licheniformis ATCC 53926

          Cell material from agar slants containing strain B.
licheniformis DSM641 was transferred by a loop and
suspended in 5 ml of medium A (Medium A: Nährbouillion from
35   Merck prepared according to the manufacturer's directions).
After intensive agitation in a Vibrox the suspension was
agitated at 30°C in a circular shaker (150 RPM, amplitude

of 2.5 cm; 24 hr). After 24 hours, 1 ml of the suspension (Optical Density = 1 at 640 nm) was incubated in 100 ml of medium A in a 500 ml Erlenmeyer shake flask for an additional 24 hours. Then it was centrifuged off at 6500

5 rpm. The centrifugate was rinsed twice with an isotonic NaCl solution and placed in enough salt water to give a cell count of approx. 10^9/ml.

About 8 ml of the suspension were transferred to a 9 cm diameter Petri dish for U.V. radiation (5-60 minutes)

10 at a distance of 27 cm from the U.V. lamp (Schutt/Göttingen) while agitating at 100 rpm. The agitation was continued during radiation to prevent settling of the cells. After the desired radiation time, preferably 6 minutes, a dilution series was made and these

15 dilutions were plated on medium-A agar plates with and without antibiotic. From the determination of CFU (Colony Forming Units) before and after mutagenic treatments, with and without antibiotics, the mortality rate can be determined as well as changes in reversion rate which

20 serves as a measurement for mutation formation. In the case of DSM 641, a mortality rate of >95% was considered to be especially desirable.

Using a replica plating technique, the colonies were transferred to Ca-caseinate-agar. The plates were

25 incubated at 39°C for 72 hours. Then at 24, 48, and 72 hours the formation of clearing zones around the colonies was measured. After measuring the colonies and the clearing zones, the strains were selected which showed a large lysis area as compared with the diameter of the

30 colony. Then individual cultures of these colonies were produced using the standard microbiological procedures (Koch's Agar Plate Procedure, Lindner's Drop Procedure, Multiple Streak Procedure). Shake flask experiments were undertaken with these isolates to determine protease

35 productivity.

Example 12

Comparison of Protease Production by DSM641 and ATCC 53926

The strains were incubated in shake flasks with 50 ml of medium B (see footnote 1 to Table III) in a 500 ml Erlenmeyer for 96 hours at 39°C and 150 RPM (amplitude of 5 cm). The protease activity was determined every 24 hours. The relative protease production is summarized in Table III.

Table III

Strain	Protease activity EPE/ml		Ratio Rel. to DSM641		Average Protease EPE/ml		Ratio Avg Rel. to DSM641	
	72[3]	96	72	96	72	96	72	96
53926[2]	4950	6702	1.36	1.32				
53926	5298	6340	1.45	1.35	5120	6270	1.35	1.33
DSM641	3950	4920	1.08	1.05				
DSM641	3650	4690	1.00	1.00	3800	4805	1.00	1.00

1. Medium B:

KH_2PO_4	2.5 g/l
$MgSO_4 \times 7H_2O$	1.0 g/l
$MnSO_4 \times 2H_2O$	0.5 g/l
$CaCl_2 \times 2H_2O$	0.2 g/l
Soy bean meal	3.0 g/l
Casein nach Hammersten	15.0 g/l
Potato starch	120.0 g/l
Amylase	0.6 g/l

The potato starch which is suspended in 1/3 of the final volume was enzymatically hydrolyzed. Separately, the casein was dissolved at 75°C in water (about 1/3 of the total volume) by addition of the KOH solution. These two solutions were combined with the salts and the total volume adjusted with water. The pH was adjusted to 7.2 and then the solution was sterilized.

2. Duplicate fermentations were performed.

3. The entries 72 and 96 refer to incubation time in hours.

Table III shows that the strain ATCC 53926 produces about 30% more protease compared to strain DSM641.

Miscellaneous Experimental Operations

1. Preparation of Plasmid DNA

Plasmid preparation (mini-prep. and CsCl/ethidium
bromide density gradient purified),agarose and acrylamide
gel electrophoresis, buffers (TE- Tris EDTA, TAE- Tris
acetate EDTA, TBE- Tris borate EDTA) and media preparation
were essentially performed as described by Maniatis, et
al., (ibid). Phenol p.a. was purchased from IBI and
equilibrated with TE-buffer prior to use. Agarose (SeaKem
GTG) and low gelling temperature agarose (SeaPlaque) were
purchased from FMC Corp., Rockland, ME.

2. Purification of Restriction Fragments

Restriction fragments were isolated by running the
sample on a preparative agarose gel. DNA was visualized by
staining with ethidium bromide and the band with the DNA
fragment to be recovered cut out with a razor blade. The
DNA was electroeluted using an IBI electroeluter,
phenolized once and precipitated with ethanol.

3. Polymerase Chain Reaction

The PCR was performed exactly as described by the
manufacturer (Cetus Corporation, Emeryville, California).
The solution containing the DNA generated by PCR was
extracted with phenol, precipitated with ethanol and
resuspended in an appropriate buffer for hydrolysis with
restriction enzymes. After hydrolysis with the appropriate
restriction enzyme, the solution was extracted with phenol
and the DNA was precipitated with ethanol. DNA prepared in
this manner was suitable for ligation. In this report the
expected length of all PCR generated DNA fragments
corresponded to the size of the DNA fragments observed
after restriction with the appropriate restriction
enzymes.

4. Restriction Analysis

Restriction enzymes, T4 DNA Ligase and bacterial
alkaline phosphatase were used as described by the
manufacturers (NEB, BRL, IBI, Boehringer).

5. Protoplast Transformation

The method of protoplast transfromation originally
optimized for B. subtilis was adapted to other Bacillus
species (Chang and Cohen (1979) Mol. Gen. Genetics 168,
5   111).   A colony of B. subtilis, B. lentus, or B.
licheniformis was inoculated into 30ml of 416 media (416
media: 2% Bactotryptone, 1% Yeast Extract, 1% NaCl,
sterilized by autoclaving) and grown overnight at 37°C in
a New Brunswick G24 tabletop shaker at 225 rpm. The next
10 morning, an appropriate volume of each culture was used to
inoculate 40 ml of 416 broth (prewarmed) in a sterile 250ml
Erlenmeyer flask.   Incubation at 37°C was continued with
shaking, until an OD_{650} between 0.4 and 0.5 was reached.
Then the cells were pelleted in a sterile 35ml
15 polypropylene Oakridge type tube, 3000rpm, 10min at 4°C.
The supernatant was removed by pouring, and the cell pellet
gently resuspended in 5ml of SMMP by inversion. SMMP was
always prepared fresh by mixing equal volumes of 2X SMM and
4X PAB (2X SMM contained: 1.0M Sucrose, 0.04M Maleic Acid,
20 0.04M $MgCl_2 \cdot 6H_2O$, pH6.5 adjusted with 10%NaOH, sterilized by
autoclaving: 4X PAB contained: 70 g of Difco Antibiotic
Medium #3 dissolved in one liter of deionized water and
sterilized by autoclaving).   The cell suspension was
transferred by pouring into a sterile 16 x 125mm round
25 bottom, screw-cap culture tube (Corning #25760).   A
lysozyme solution, 10mg/ml in SMMP was prepared fresh, and
200 μl added to the cell suspension. The tubes were placed
on the platform of a G24 New Brunswick shaker, and
incubated at 37°C with very gentle shaking (25-30 rpm).
30 Protoplast formation generally took 40-60 minutes for B.
licheniformis, and 20-40 minutes for B. lentus and B.
subtilis.   To determine the degree of protoplasting, a
sample of the cell suspension was examined using the phase
contrast microscope.   Following the lysozyme treatment,
35 10ml of SMMP was added to the protoplast suspension and the
protoplasts were pelleted by centrifugation at 2000 rpm for
10 minutes at room temperature. The supernatant was

carefully removed before resuspending the protoplast pellet
in SMMP (3ml of SMMP for each 40 ml of initial cell
culture). In each transformation reaction, 0.5ml of
protoplasted cells was transferred into a sterile, plastic
5   16 x125mm round bottom culture tube (Corning #256760),
followed by the addition of one to four micrograms of DNA
in a volume of 60μl or less, and 1.5 ml of 40% polyethylene
glycol (PEG, Sigma Chemical Company product #P-3640).
After gentle mixing of the tubes by inversion they were
10   allowed to sit at room temperature for 3-4 minutes, and
then the PEG was diluted by the addition of 5ml of SMMP +
2% BSA (Bovine Serum Albumin). After mixing by inversion,
the protoplasts were pelleted by centrifugation at 2000 rpm
for 10 minutes at room temperature, and the supernatant was
15   carefully poured off before resuspending the pellet in
1.0ml of SMMP+BSA. The cells were then incubated at 37°C
for 90 minutes with shaking (New Brunswick G24 table top
shaker, 150 rpm) to allow for expression of plasmid encoded
genes, and to partially regenerate cell walls. Selective
20   regeneration medias were prepared with the appropriate
antibiotic. The CR5 regeneration medium was used for all
<u>Bacillus</u> strains when selecting for transformants
containing plasmids encoding Km^R (Puyet <u>et al.</u>(1987) FEMS
Microbiology Letters <u>40</u>, 1). The DM3 regeneration medium
25   was originally used to select all <u>Bacillus</u> strains
transformed by plasmids encoding Tc^R (Chang and Cohen
(ibid)). However, <u>B</u>. <u>lentus</u> protoplasts were discovered to
regenerate inefficiently on this succinate based media.
Therefore, #451 regeneration medium which used mannitol as
30   an osmotic stabilizer was tested (Bourne and Dancer (1986)
J. of Gen. Micro. <u>132</u>, 251). All of the <u>Bacillus</u> strains
were found to regenerate well on the mannitol media,
although the level of tetracycline needed for each strain
was different. The selective levels of tetracycline for <u>B</u>.
35   <u>subtilis</u>, <u>B</u>. <u>lentus</u>, and ATCC 53926 were 65 μg/ml, 15
μg/ml, and 25 μg/ml, respectively. Each transformation
reaction was plated onto 5 regeneration plates and

incubated 1-2 days at 37°C. Potential transformants were picked onto Luria Agar containing the appropriate antibiotic, and when necessary 1% skim milk to detect protease activity. To check strain identity, Spizizens's

5 minimal salts medium with and without the correct nutritional additives were used.

6. Bacterial Strains

<u>Escherichia coli</u> Strains:

1. <u>E</u>. <u>coli</u> NM522 is hsdΔ5,Δ(lac-pro), [F', pro⁺,

10 lacI^qZΔM15]. NM522 is restriction minus and modification minus (Pharmacia, Piscataway, N.J.)

2. <u>E</u>. <u>coli</u> GM33 (dam3) is dam-methylase minus (Marinus, M. G. and Morris, N. R., (1974) J. Mol. Biol. <u>85</u>, 309).

3. <u>E</u>. <u>coli</u> DH5∝ is F-φ80dlacZΔM15, Δ(lacZYA-argF)U169,

15 recA1, endA1, hsdR17(r_k^-,m_k^+), supE44, lambda-, thi-1, gyrA, relA1 (competent frozen cells, BRL, Gaithersburg, MD).

4. <u>E</u>. <u>coli</u> HB101 is F-, hsdS20 (r_B^-, m_B^-), supE44, ara14, glaK2, lacYi, proA2, rpsL20(str^R), xyl15, leu, mtl1, lambda-, recA13.

20   ### 7. Bacillus Strains:

1. <u>B</u>. <u>subtilis</u> DB104 has the genotype: his, nprR2, nprE18, and aprA3. This strain was obtained from Dr. Roy Doi at the University of California at Davis through a licensing agreement, and has only been used for research purposes.

25 This strain is deficient in the production of neutral and alkaline protease.

2. <u>B</u>. <u>licheniformis</u> ATCC 53926 is an industrial production strain developed by Henkel KGaA, Düsseldorf, West Germany.

30 3. <u>B</u>. <u>lentus</u> DSM 5483 is a natural isolate of a soil sample from West Germany.

8. Cloning Vectors

1. pUC19: pUC19 is a small <u>E</u>. <u>coli</u> plasmid (2686 bp) containing parts of pBR322 and M13mp19. It carries a 54 bp

35 multiple cloning site with recognition sites for 13 different restriction endonucleases. It has been constructed by C. Yanisch-Perron <u>et</u> <u>al</u>. ((1985), Gene <u>33</u>,

103), and can be purchased from New England Biolabs, Inc., Beverly, MA.

2. pTZ18R: pTZ18R (Mead, D.A. et al. (1986) Protein Engineering, 1, 67) is a multifunctional phage plasmid (phasmid) vector. It contains origins of replication of the E. coli vector pBR322 and of the E. coli phage f1, allowing single-stranded DNA to be produced. It contains the multiple cloning site of plasmid pUC18.   It can be purchased from Pharmacia LKB Biotechnology, Inc., Piscataway, NJ.

3. pTZ19R: pTZ19R is homologous to pTZ18R but contains the multiple cloning site of plasmid pUC19.

4. pCP13:   pCP13 is a broad host range cosmid vector derived from plasmid pLAFR1, which has been previously characterized (Friedman, et al.,(1982) Gene 18, 289). Plasmid pCP13 is 23 kb in size, and encodes resistance to tetracycline and kanamycin.   It contains multiple unique restriction sites suitable for cloning, can be mobilized, but is not self-transmissible.

5. pC50:   pC50 is a derivative of the Bacillus plasmid pBC16. It contains a 2 kb DNA fragment encoding the DSM641 protease cloned into the BamHI site of pBC16.

6. pC51:   pC51 is a second generation derivative of plasmid pC50.   The 1540 bp AvaI/SstI fragment from pC50 which encodes an ATCC 53926 alkaline protease, was first cloned into the E. coli plasmid pUC19 between the XmaI and SstI sites to form plasmid pH9.   The ATCC 53926 protease gene was then removed from pH9 on an EcoRI/BamHI fragment and cloned into plasmid pBC16 between the EcoRI and BamHI sites to form plasmid pC51.

7. pUB110:   pUB110 is a 4548 bp plasmid encoding resistance to kanamycin, which was originally isolated from Staphylococcus aureus by R.W. Lacey ((1974) J. Med. Microbiol., 7, 285).

8. pKO110:   pKO110 is a derivative of pUB110 in which the 788 bp EcoR1/BamHI fragment has been removed, and replaced by a fragment from pUC19 containing the polylinker region.

9. pBC16: pBC16 has an approximate size of 4250 bp, and encodes resistance to tetracycline. This plasmid was originally isolated from Bacillus cereus and transformed into B. subtilis (Bernhard, K., et. al., (1978) J. Bacteriol.133:897).

10. pC194: pC194 is a 2910 basepair plasmid encoding resistance to chloramphenicol which was isolated from S. aureus and transformed into B. subtilis. (Iordanescu, S., et. al., (1978) Plasmid 1:468) and (Erlich, S.D. (1977), PNAS USA 74:1680).

11. pH70: pH70 is a derivative of plasmid pUB110. The ATCC 53926 alkaline protease gene was removed from plasmid pH9 (see plasmid pC51) on an EcoRI/BamHI DNA fragment and cloned into plasmid pUB110 between the EcoRI and BamHI sites to form plasmid pH70.

What is claimed is:
1.   A composition comprising a mature alkaline proteolytic enzyme from <u>Bacillus lentus</u> DSM 5483 having the amino acid sequence from about amino acid residue 112 to about amino acid residue 380 substantially as shown in Figure 1 and at least one other proteolytic enzyme from <u>Bacillus licheniformis</u> ATCC 53926.

2.   A hybrid plasmid capable of replicating in <u>Bacillus</u> containing a DNA sequence comprising in the direction of transcription a regulatory region, said regulatory region comprising in the order of transcription a <u>Bacillus licheniformis</u> ATCC 53926 alkaline protease promoter, ribosome binding site, and initiation codon operably linked to a pre-pro region, said pre-pro region operably linked to a mature region encoding for said proteolytic enzyme from <u>Bacillus lentus</u> DSM 5483 of claim 1.

3.   The hybrid plasmid of claim 2 further comprising a <u>Bacillus licheniformis</u> ATCC 53926 alkaline protease transcription terminator operably linked to said mature region encoding for said proteolytic enzyme from <u>Bacillus lentus</u> DSM 5483 of claim 1.

4.   The hybrid plasmid of claim 2 further comprising a carboxy terminal portion of the <u>Bacillus licheniformis</u> ATCC 53926 alkaline protease gene and its transcriptional terminator operably linked to said mature region encoding for said proteolytic enzyme from <u>Bacillus lentus</u> DSM 5483 of claim 1.

5.   An isolated DNA sequence coding for the pre-pro-mature form of said proteolytic enzyme from <u>Bacillus lentus</u> DSM 5483 of claim 1.

6.   The hybrid plasmid pCB55C having the cleavage map substantially as shown in Figure 13.

7.   The hybrid plasmid pCB56C having the cleavage map substantially as shown in Figure 14.

8.   The hybrid plasmid pCB58C having the cleavage map substantially as shown in Figure 15.

9.   The hybrid plasmid pCB75C having the cleavage map substantially as shown in Figure 16.

10.   The hybrid plasmid pCB78C having the cleavage map substantially as shown in Figure 17.

11.   The hybrid plasmid pCB50N having the cleavage map substantially as shown in Figure 18.

12.   The hybrid plasmid pCB51N having the cleavage map substantially as shown in Figure 19.

13.   The hybrid plasmid pCB53N having the cleavage map substantially as shown in Figure 20.

14.   The hybrid plasmid pCB70N having the cleavage map substantially as shown in Figure 21.

15.   The hybrid plasmid pCB73N having the cleavage map substantially as shown in Figure 22.

16.   A transformed Bacillus host cell comprising a hybrid plasmid of claim 6.

17.   A transformed Bacillus host cell comprising a hybrid plasmid of claim 7.

18. A transformed <u>Bacillus</u> host cell comprising a hybrid plasmid of claim 8.

19. A transformed <u>Bacillus</u> host cell comprising a hybrid plasmid of claim 9.

20. A transformed <u>Bacillus</u> host cell comprising a hybrid plasmid of claim 10.

21. A transformed <u>Bacillus</u> host cell comprising a hybrid plasmid of claim 11.

22. A transformed <u>Bacillus</u> host cell comprising a hybrid plasmid of claim 12.

23. A transformed <u>Bacillus</u> host cell comprising a hybrid plasmid of claim 13.

24. A transformed <u>Bacillus</u> host cell comprising a hybrid plasmid of claim 14.

25. A transformed <u>Bacillus</u> host cell comprising a hybrid plasmid of claim 15.

26. The transformed <u>Bacillus</u> host cell of claim 16 wherein said host cell is <u>Bacillus licheniformis</u> ATCC 53926.

27. The transformed <u>Bacillus</u> host cell of claim 17 wherein said host cell is <u>Bacillus licheniformis</u> ATCC 53926.

28. The transformed <u>Bacillus</u> host cell of claim 18 wherein said host cell is <u>Bacillus licheniformis</u> ATCC 53926.

29. The transformed <u>Bacillus</u> host cell of claim 19 wherein said host cell is <u>Bacillus licheniformis</u> ATCC 53926.

30.   The transformed _Bacillus_ host cell of claim 20 wherein said host cell is _Bacillus_ _licheniformis_ ATCC 53926.

31.   The transformed _Bacillus_ host cell of claim 21 wherein said host cell is _Bacillus_ _licheniformis_ ATCC 53926.

32.   The transformed _Bacillus_ host cell of claim 22 wherein said host cell is _Bacillus_ _licheniformis_ ATCC 53926.

33.   The transformed _Bacillus_ host cell of claim 23 wherein said host cell is _Bacillus_ _licheniformis_ ATCC 53926.

34.   The transformed _Bacillus_ host cell of claim 24 wherein said host cell is _Bacillus_ _licheniformis_ ATCC 53926.

35.   The transformed _Bacillus_ host cell of claim 25 wherein said host cell is _Bacillus_ _licheniformis_ ATCC 53926.

36. A method for producing the enzyme of claim 1 comprising cultivating a _Bacillus_ _licheniformis_ ATCC 53926 strain which has been transformed by a plasmid having a gene encoding for said enzyme in a nutrient medium and recovering said enzyme.

37. The method of claim 36 wherein said plasmid is pCB55C.

38. The method of claim 36 wherein said plasmid is pCB56C.

39. The method of claim 36 wherein said plasmid is pCB58C.

40. The method of claim 36 wherein said plasmid is pCB75C.

41. The method of claim 36 wherein said plasmid is pCB78C.

42. The method of claim 36 wherein said plasmid is pCB50N.

43. The method of claim 36 wherein said plasmid is pCB51N.

44. The method of claim 36 wherein said plasmid is pCB53N.

45. The method of claim 36 wherein said plasmid is pCB70N.

46. The method of claim 36 wherein said plasmid is pCB73N.

47. A composition comprising a mature alkaline proteolytic enzyme having the amino acid sequence substantially as shown in Figure 29 and at least one other proteolytic enzyme from Bacillus licheniformis ATCC 53926.

48. A biologically pure culture of Bacillus licheniformis having all the characteristics of the strain deposited under accession number ATCC 53926.

49. A biologically pure culture of Bacillus lentus strain having all the characteristics of the strain deposited under accession number DSM 5483.

50. A composition comprising an alkaline proteolytic enzyme of claim 1 in combination with a surfactant.

51. A composition of claim 50 wherein said surfactant is selected from the group consisting of a linear alkyl benzene sulfonate, an ethoxylated linear alcohol, an alkyl ethoxylated sulfate, a sulfated linear alcohol, or combinations thereof.

```
          5         10        15        20        25        30
          |         |         |         |         |         |
   1 M K K P L G K I V A S T A L L I S V A F S S I A S A A E E
  31 A K E K Y L I G F N E Q E A V S E F V E Q V E A N D E V A I
  61 L S E E E E V E I E L L H E F E T I P V L S V E L S P E D V
  91 D A L E L D P A I S Y I E E D A E V T T M A Q S V P W G I S
 121 R V Q A P A A H N R G L T G S G V K V A V L D T G I S T H P
 151 D L N I R G G A S F V P G E P S T Q D G N G H G T H V A G T
 181 I A A L N N S I G V L G V A P S A E L Y A V K V L G A D G R
 211 G A I S S I A Q G L E W A G N N G M H V A N L S L G S P S P
 241 S A T L E Q A V N S A T S R G V L V V A A S G N S G A S S I
 271 S Y P A R Y A N A M A V G A T D Q N N R A S F S Q Y G A G
 301 L D I V A P G V N V Q S T Y P G S T Y A S L N G T S M A T P
 331 H V A G A A A L V K Q K N P S W S N V Q I R N H L K N T A T
 361 S L G S T N L Y G S G L V N A E A A T R
```

Amino acid composition
----

54 A	0 C	8 H	5 M	21 T
9 R	12 Q	19 I	6 F	3 W
24 N	28 E	30 L	17 P	9 Y
11 D	36 G	10 K	43 S	36 V

Number of residues: 380
Molecular weight (MW): 38951

FIG. 1

FIG. 2-1

```
              10        20        30        40        50
               |         |         |         |         |
   1 AAGCTTCTAGAGATCTTCCATACCTACCAGTTCTGCGCCTGCAGCAATGG
  51 CAACAACGTCGCGGATCATTACAAAGACGATTGCCAAGGCCGCTAACTGA
 101 ATACTGCTAGCTGTGTACCACTGTAAAGCGAAAGGTGCGTCTAAGTAATA
 151 AGGCCGAACCTTAAATTAAATTCCCTCGTTTGCCCACACAGGCTTTTTAG
 201 GCGTGTGTATTTTGTTATGCCTAGTAGAATCAAAATAGACATAAGAACTG
 251 GAGGACTTATTATATGCATGAACAATTGAGACAAACGACACAACCAACGA
 301 TTATTGTTGGTGTGGAGTTCGAAAAAGACACGGATTTCCACTATTCGATG
 351 GAAGAATTGAACAATTTGGCCGATCGCTTGATTTGCAGGTTGTCGACAGC
 401 TCACACAAAAGCTGCCTGTACCAAATCAAGCGACATATATTGGTGCTGGC
 451 AAAGCGTCTGAGCTTTCCTGATGTGTGAATCGCTTGGGGGCTACGCTCGT
 501 TGTGTTTAATGACGAGCTCTCACCTTCACAAATCCGCAATCTTGAAAAAT
 551 TGCTGGAAGTAACCGTTTATGATCGGACCATGTTGATTTTAGATATTTTT
 601 GGCGAAAGGGCGAAGACGAAAGAAGCTCAACTCCAAGTAGAAATGGCGCG
 651 CTGCCGCTATTTGTTCGCACGGCTCGTCGGCATGCGCGCTTCATTAAGCA
 701 GGCAAGGCGGCCGGCACTGGCTTGGCCAACCGCGGCGCCGGGGAAACAAA
 751 GTTGGAGCTCGATCGTCGTAAAATTGAAACACGTATTCATGCGTTGGAAA
 801 AAGAGCTGGAAGACGTTGTCAAACGACGCGAGTTACAGCGCAAACGCCGG
 851 AAAAAACAAGCGATGCCGGTCGTTGCCTTAGTTGGCTATACAAATGCAGG
 901 GAAATCTTCGCTGCTGAATGCGCTGTTGGACGATGCCGAGGAAAAGCGTG
 951 TGTTGGAAAAAGATATGTTGTTTGCCACTCTTGATACGTCTGTGCGTAAA
1001 GTGGAACTTGACAAAAACCATTCTGTTTTATTAGCGGATACGGTTGGCTT
1051 TGTCTCTAAATTGCCAACACACCTCGTTAAAGCATTCCGCTCAACATTGG
1101 AAGAAGCTCGGGAAGCCGATTTGCTACTGCATGTCGTCGATTATTCAAAT
1151 GAACGCCATCGCGAAATGGCAAAGACGACAAATGAAACACTCCAGGCAAT
1201 GGAAATCGATCGCCCGATGATTTATGTTTACAACAAAATGGATCGAGTGA
1251 AAGACGCGTTTCCTCAAGCGCATGGGCACGAGCTGTTTATATCAGCTAAG
1301 GCTAAACAAGGGCTTGATTTATTAGCACAGAAAATAGCAAGCTATGTTTT
1351 TCAAGATTTTGAAAAACATCTGTTCATCATTCCTTATCGTGACGGGGAGG
1401 CGGCTGCTTATTTAAACAACCATGCCCATGTTCACACACAGCGTGCTGAG
1451 GAGGACGGCTGGCATATCGTTGCCGATTTGCATGAACGAGACTTGAAACG
1501 GGTTGAAAGCTACTGTGTTTCAAAAGAACGATAATGAAAAAAGCCATTTG
1551 AATGCTTCTTGTTCAAATGGCTTTTTGGCGACTATGGTAGACAGATGAAC
1601 ACTTGTTTCGCTGTTTTACGACAAAGATCATCCTGCCTGTTACGCGTTTT
1651 TTAAATCCGTTTTCGCACGTTCAATTGTCGCCGAGTCGTACCAGTCGCTG
1701 TAAGTGAGAATATGTTTAGAAAGCCGCGTATTTAAGCGCAGTCTTTTTCG
1751 TTCTGTACTGGCTGGTTTGTGGACAGTTTCCATACCCATCGGCCTCCTTT
1801 TATTTGTAGCTTTCCCCACTTGAAACCGTTTTAATCAAAAAAGAAACGGG
1851 AAGATTCTGTTAACTTAACGTTAATATTTGTTTCCCAATAGGCAAATCTT
1901 TCTAACTTTGATACGTTTAAACTACCAGCTTGGACAAGTTGGTATAAAAA
1951 TGAGGAGGGAACCGAATGAAGAAACCGTTGGGGAAAATTGTCGCAAGCAC
2001 CGCACTACTCATTTCTGTTGCTTTTAGTTCATGCATCGCATCGGCTGCTG
2051 AGGAAGCAAAAGAAAAATATTTAATTGGCTTTAATGAGCAGGAAGCTGTC
2101 AGTGAGTTTGTAGAACAAGTAGAGGCAAATGACGAGGTCGCCATTCTCTC
2151 TGAGGAAGAGGAAGTCGAAATTGAATTGCTTCATGAGTTTGAAACGATTC
2201 CTGTTTTATCCGTTGAGTTAAGCCCAGAAGATGTGGACGCGCTTGAACTT
2251 GATCCAGCGATTTCTTATATTGAAGAGGATGCAGAAGTAACGACAATGGC
2301 GCAATCAGTGCCATGGGGAATTAGCCGTGTGCAAGCCCCGGCTGCCCATA
2351 ACCGTGGATTGACAGGTTCTGGTGTAAAAGTTGCTGTCCTCGATACAGGT
```

```
2401 ATTTCCACTCATCCAGACTTAAATATTCGTGGTGGCGCTAGCTTTGTACC
2451 AGGGGAACCATCCACTCAAGATGGGAATGGGCATGGCACGCATGTGGCCG
2501 GGACGATTGCTGCTTTAAACAATTCGATTGGCGTTCTTGGCGTAGCGCCT
2551 AGTGCGGAACTATACGCTGTTAAAGTTTTAGGAGCCGACGGTAGAGGTGC
2601 AATCAGCTCGATTGCCCAAGGGTTGGAATGGGCAGGGAACAATGGCATGC
2651 ACGTTGCTAATTTGAGTTTAGGAAGCCCTTCGCCAAGTGCCACACTTGAG
2701 CAAGCTGTTAATAGCGCGACTTCTAGAGGCGTTCTTGTTGTAGCGGCATC
2751 TGGGAATTCAGGTGCAAGCTCAATCAGCTATCCGGCCCGTTATGCGAACG
2801 CAATGGCAGTCGGAGCTACTGACCAAAACAACAACCGCGCCAGCTTTTCA
2851 CAGTATGGCGCAGGGCTTGACATTGTCGCACCAGGGGTAAACGTGCAGAG
2901 CACATACCCAGGTTCAACGTATGCCAGCTTAAACGGTACATCGATGGCTA
2951 CACCTCATGTTGCAGGTGCAGCAGCCCTTGTTAAACAAAAGAACCCATCT
3001 TGGTCCAATGTACAAATCCGCAACCATCTAAAGAATACGGCAACGAGCTT
3051 AGGAAGCACGAACTTGTATGGAAGCGGACTTGTCAATGCAGAAGCGGCAA
3101 CACGCTAATCAATAAAAAAACCGTGTGCGCTTAAAGGGCACAGCTTTTTT
3151 TGTGTATGAATCGAAAAAAGAGAACAGATCGCAGGTCTCAAAAATCGAGC
3201 GTAAAGGGGTTGTTTAAAGCTCTTTACGCTCGCAGGTCTTATCGCTATACA
3251 ATGGAAAATTCACGTCTTTTGACTTTCATGGCATATATTTATTTAAGTAT
3301 TCGTTTGCTTTTTTCGTACTCTCCGTTTTTCTGGTACCATTGCGCCAGCTC
3351 AATTGCATAGTGGACTGGTTCTTCTTTATTATCAAGCTT
```

Total number of bases is: 3389.
DNA sequence composition: 949 A; 716 C; 820 G; 904 T;

FIG. 2-2

FIG. 3
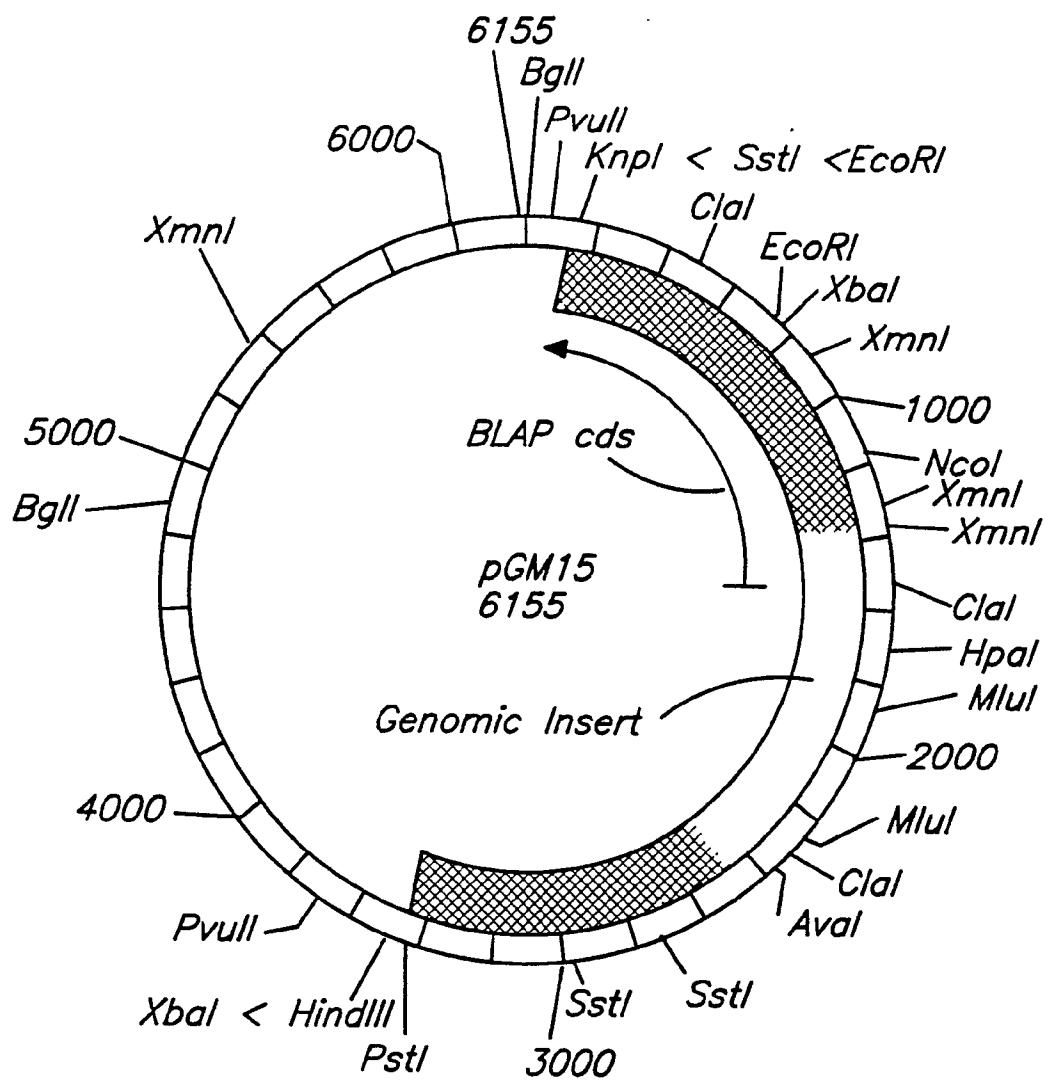
FIG. 4
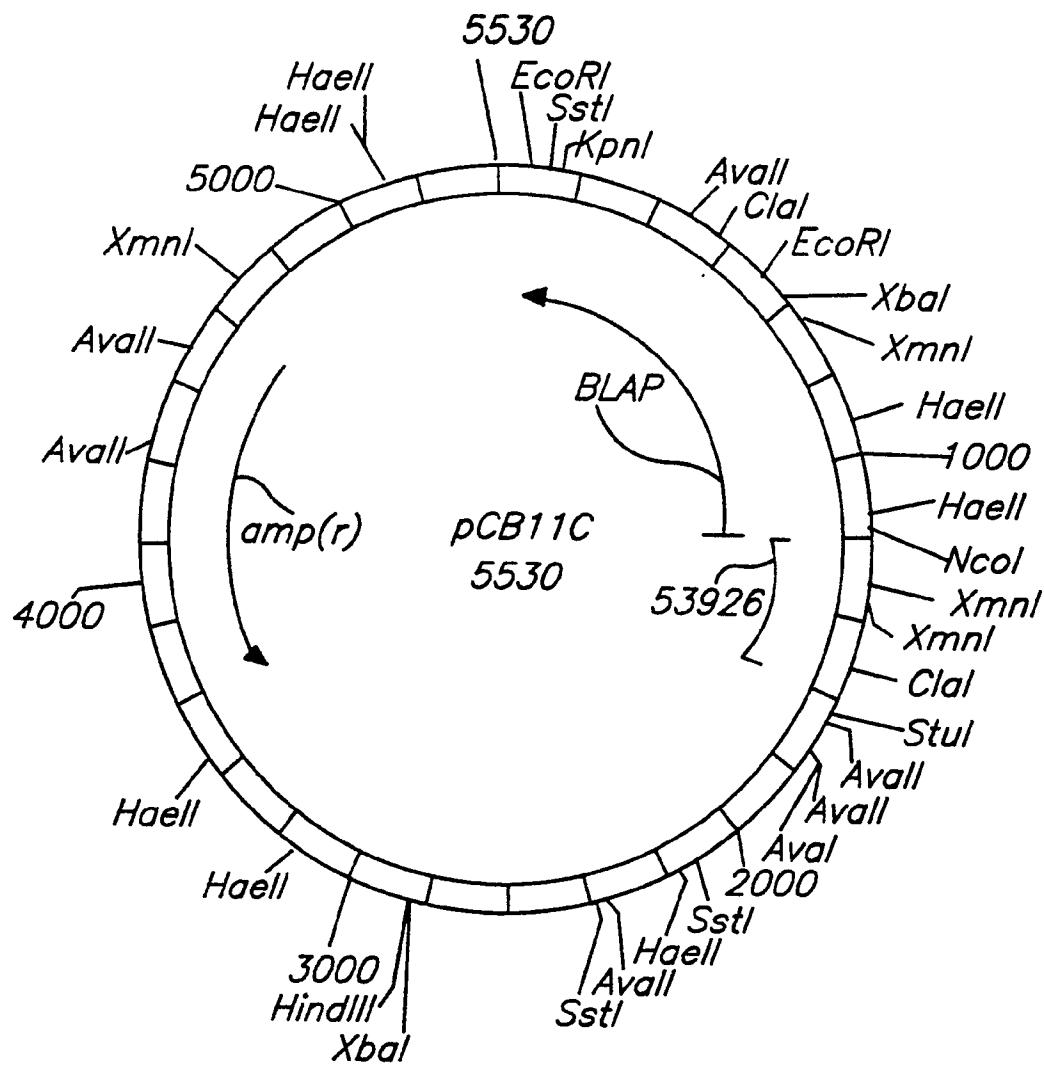
FIG. 5
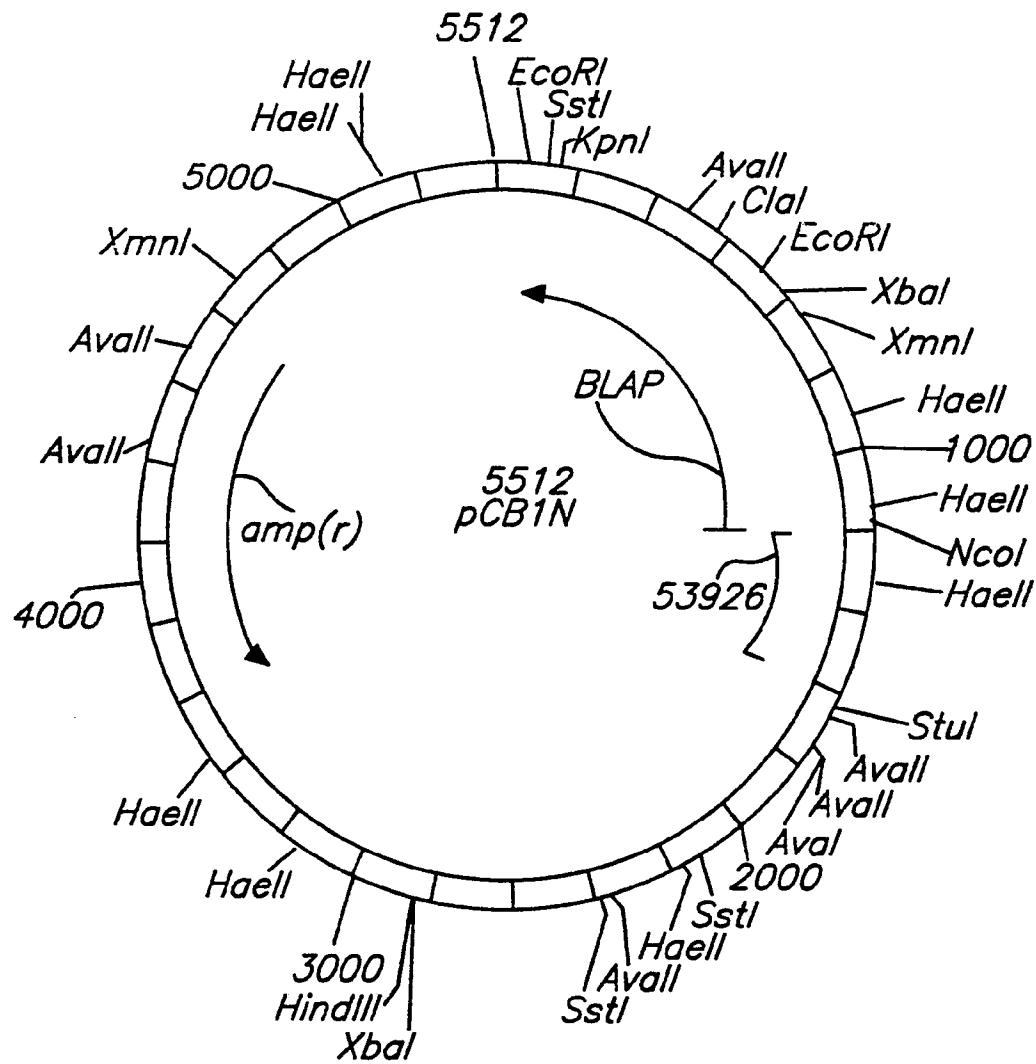
FIG. 6
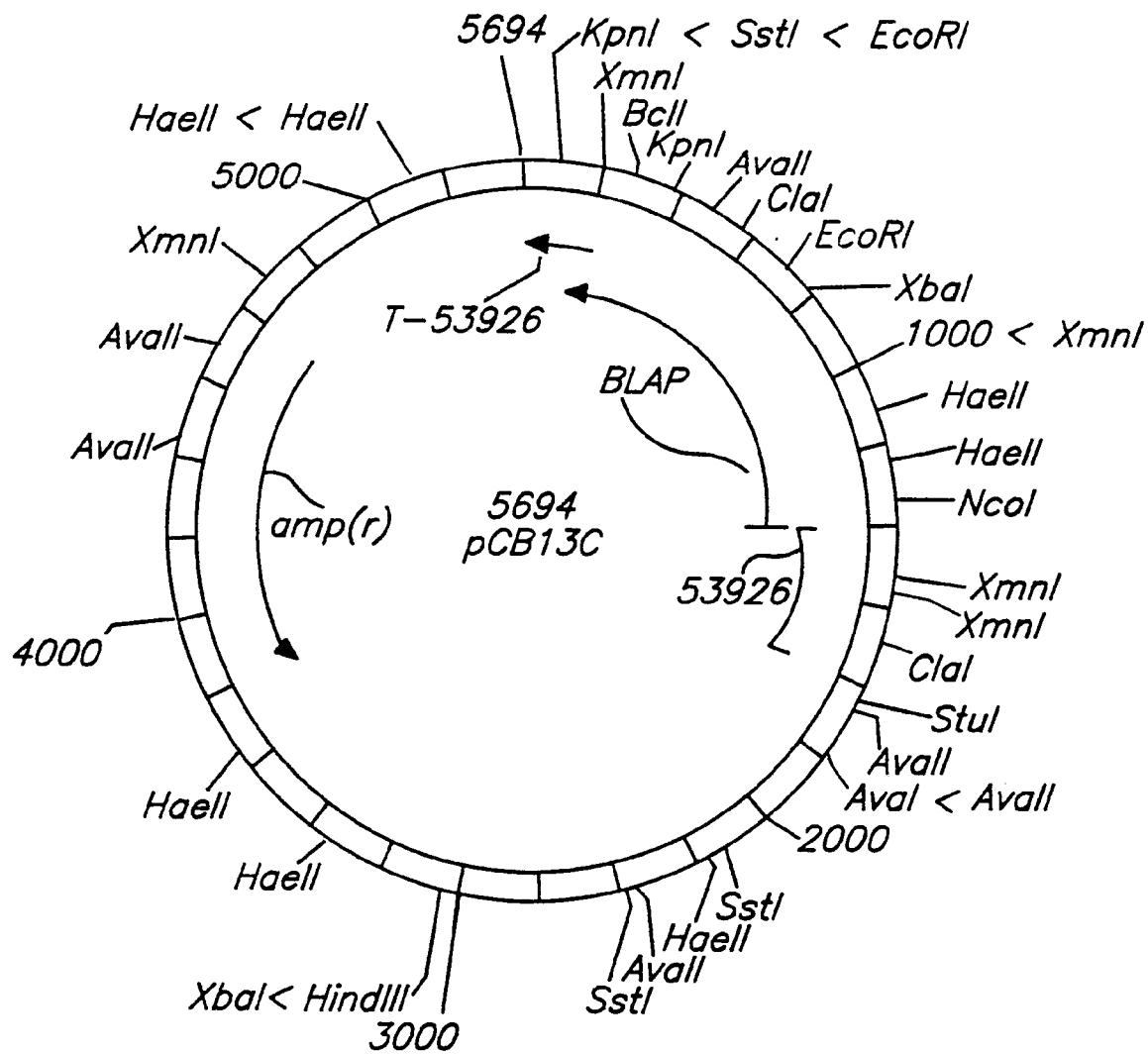
FIG. 7
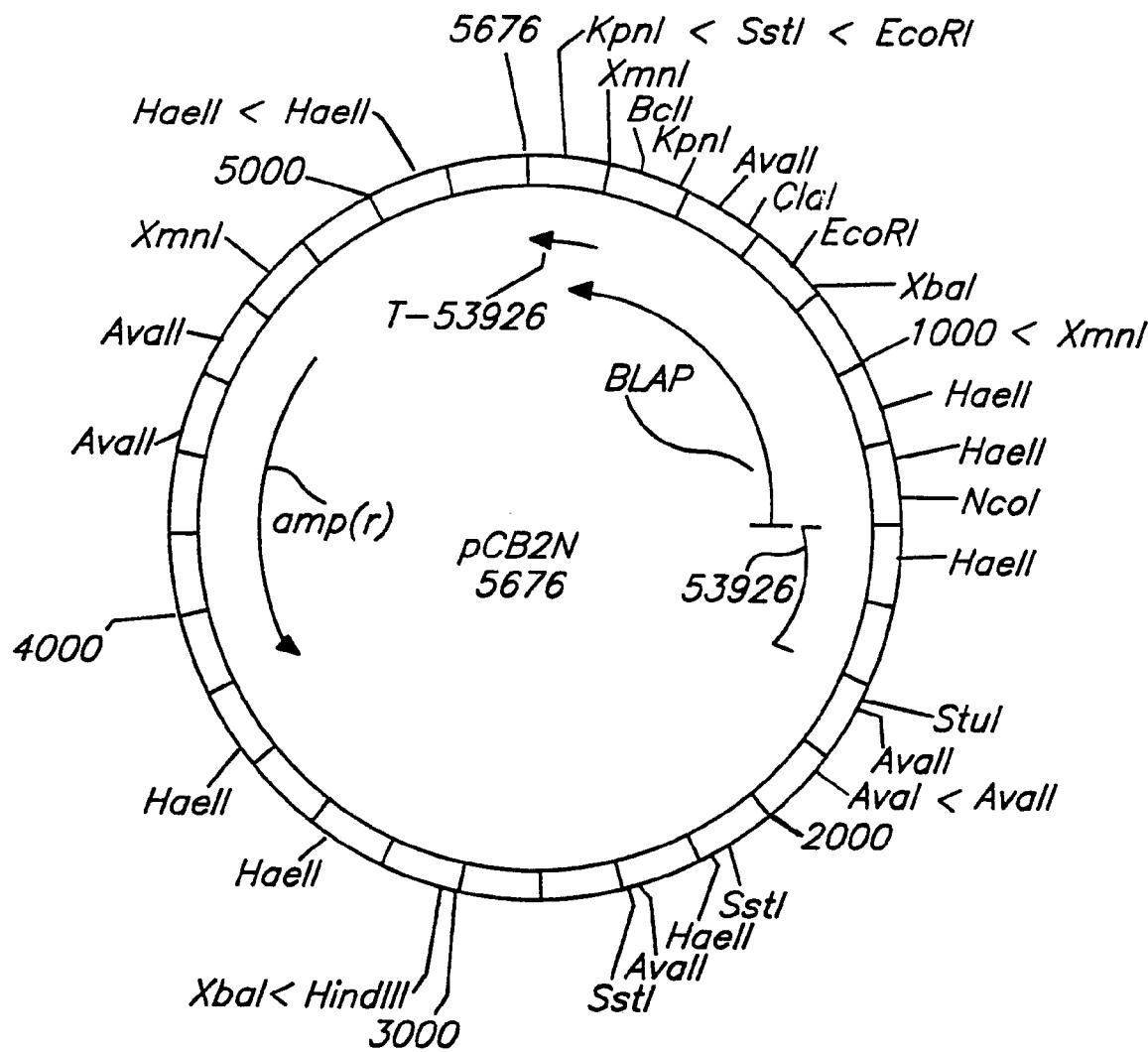
```
             80                      100
#1  5' - GGCCCTCGGGACCTCTTTCCC - 3'  (53926, top strand)
              Ava I


             651                     633
#2  5' - GGAATGCCccAtGGAACGG - 3'   (53926, bottom strand)
                Nco I


             390                         361
#3  5' - GCGGAATCGaTcgATGCCATCGTGAACACG - 3'   (53926, bottom strand)
                Cla I
```

FIG. 8

FIG. 9
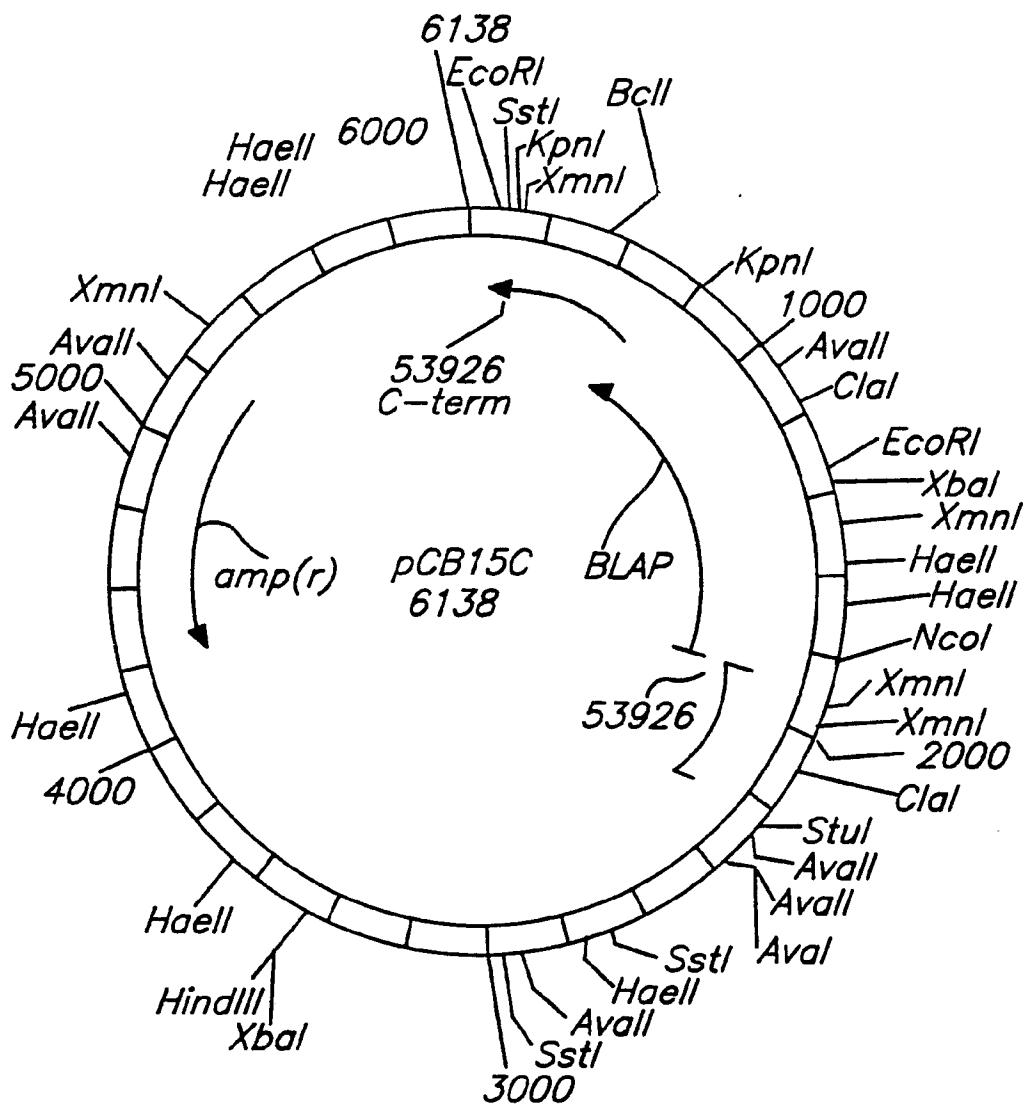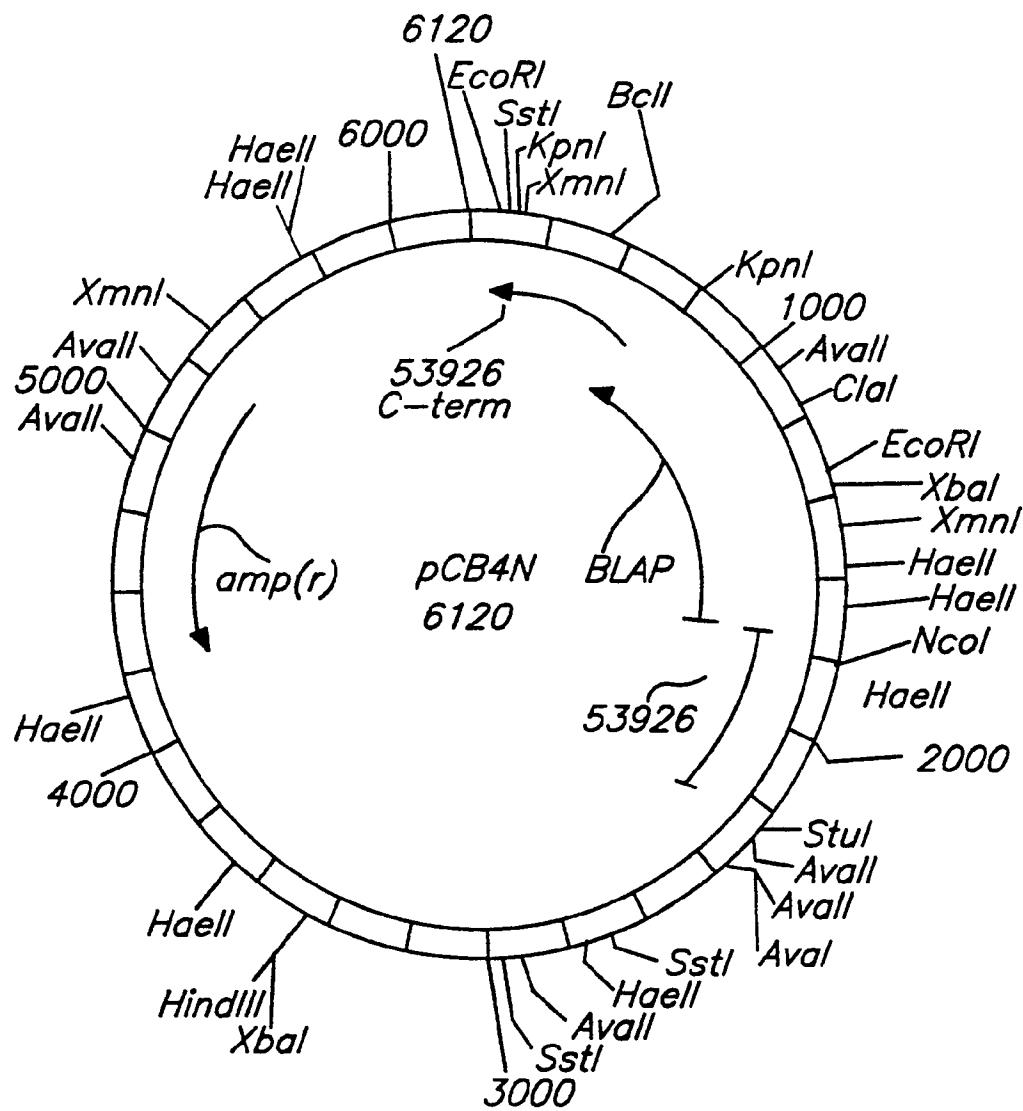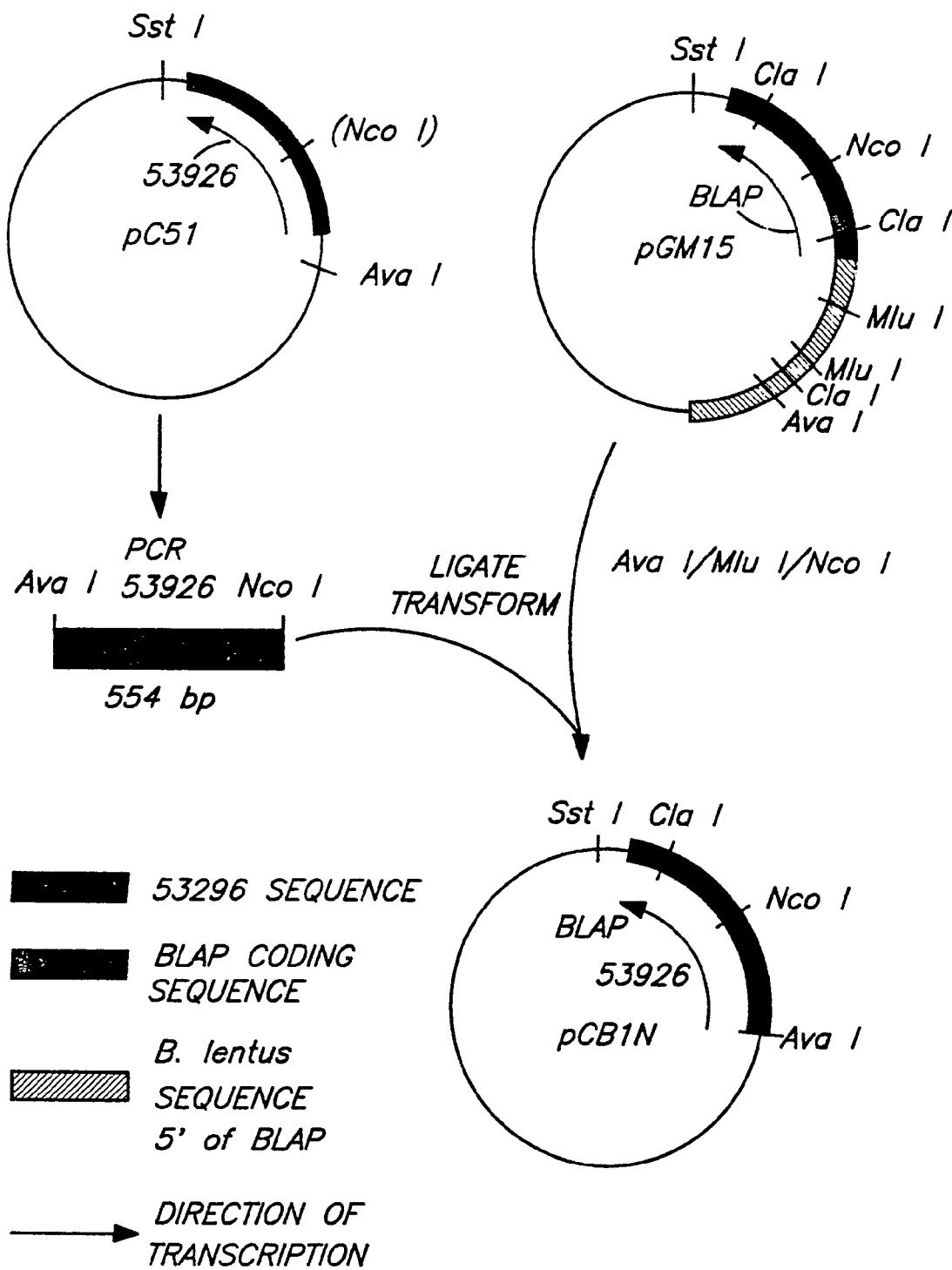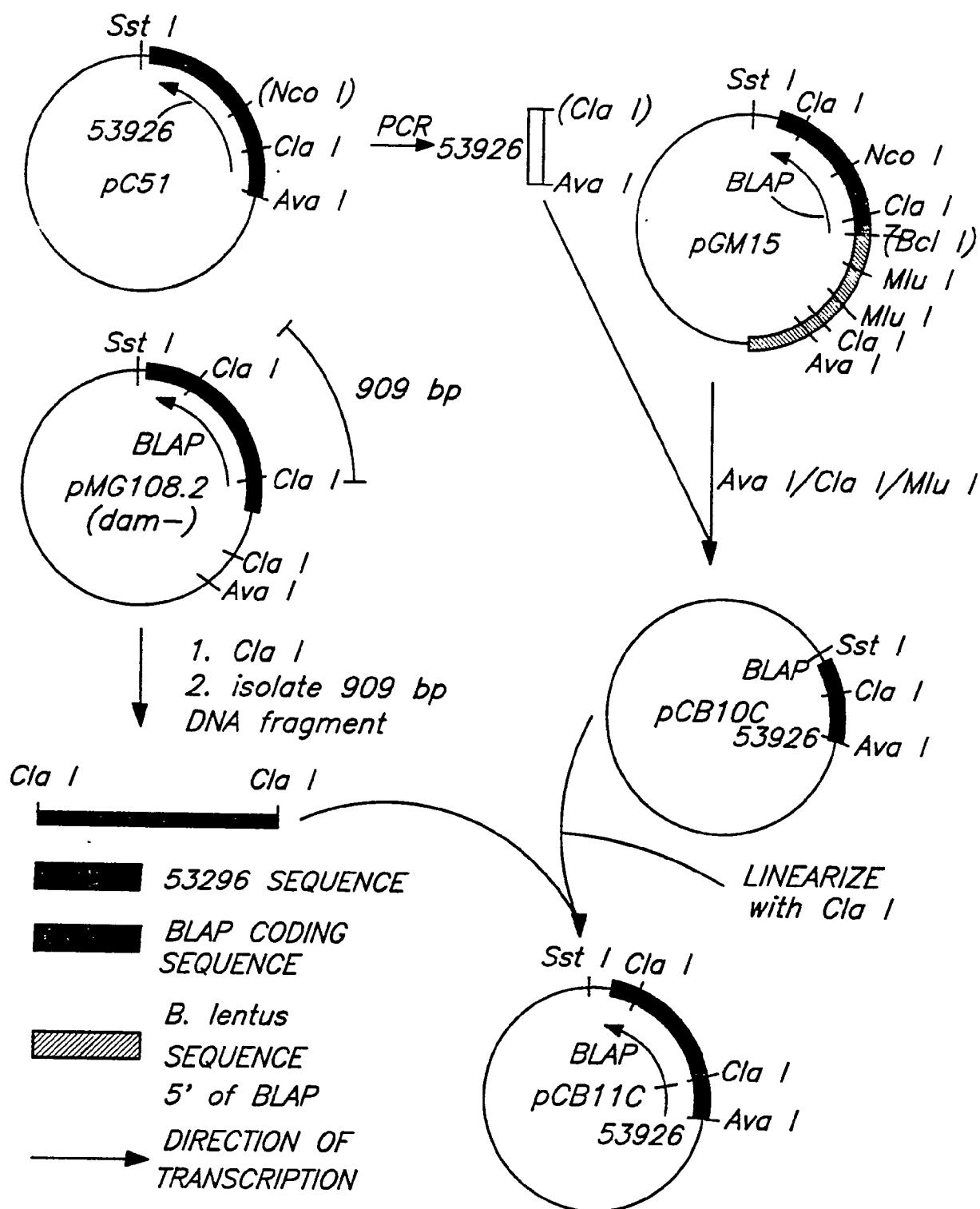
FIG. 10

FIG. 11

Sst I

53926
pC51

(Nco I)

Ava I

Sst I   Cla I

Nco I

BLAP
pGM15

Cla I

Mlu I

Mlu I
Cla I
Ava I

PCR
Ava I   53926   Nco I

554 bp

LIGATE
TRANSFORM

Ava I/Mlu I/Nco I

Sst I   Cla I

Nco I

BLAP
53926
pCB1N

Ava I

53296 SEQUENCE

BLAP CODING
SEQUENCE

B. lentus
SEQUENCE
5' of BLAP

DIRECTION OF
TRANSCRIPTION

FIG. 12

FIG. 13
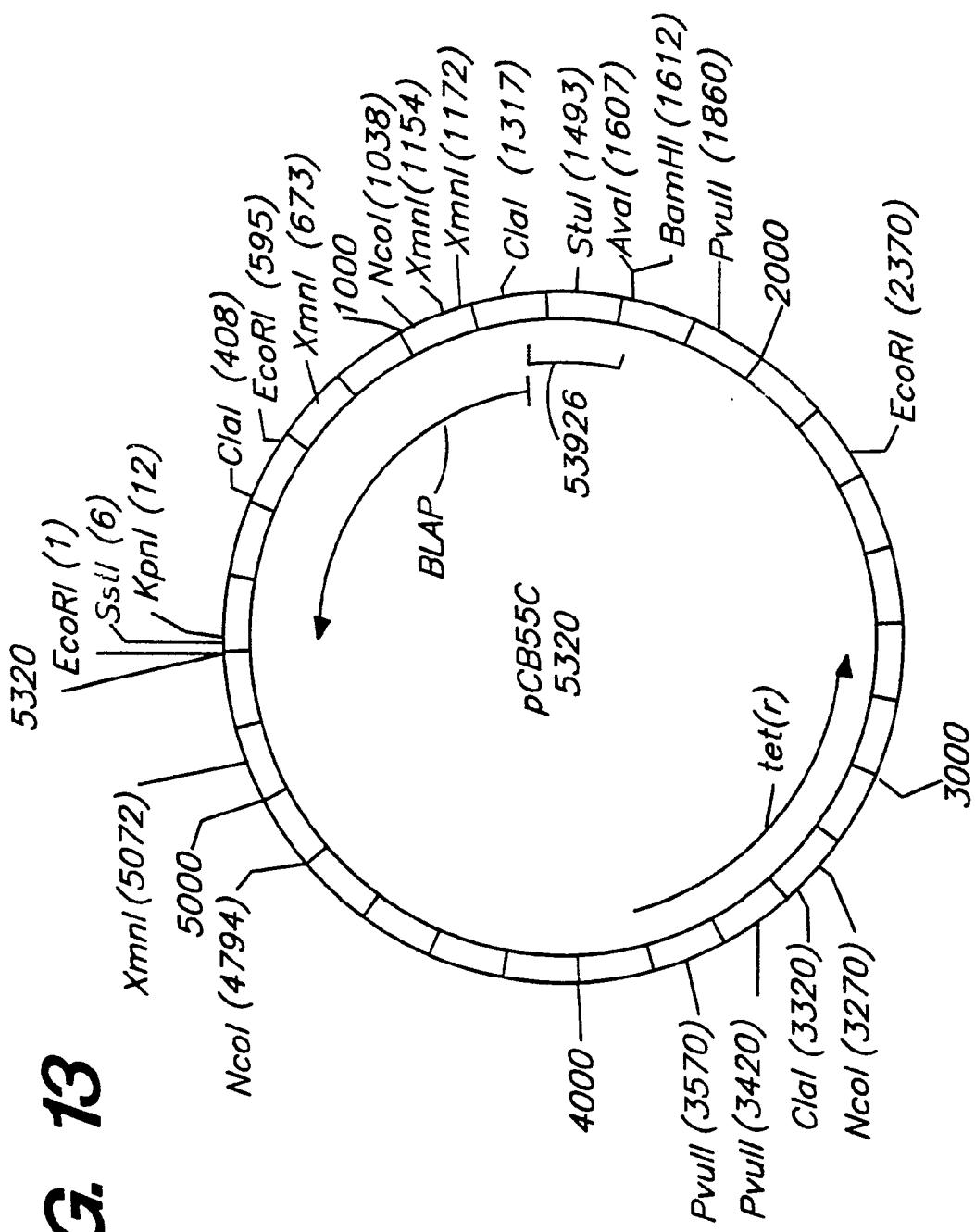
FIG. 14
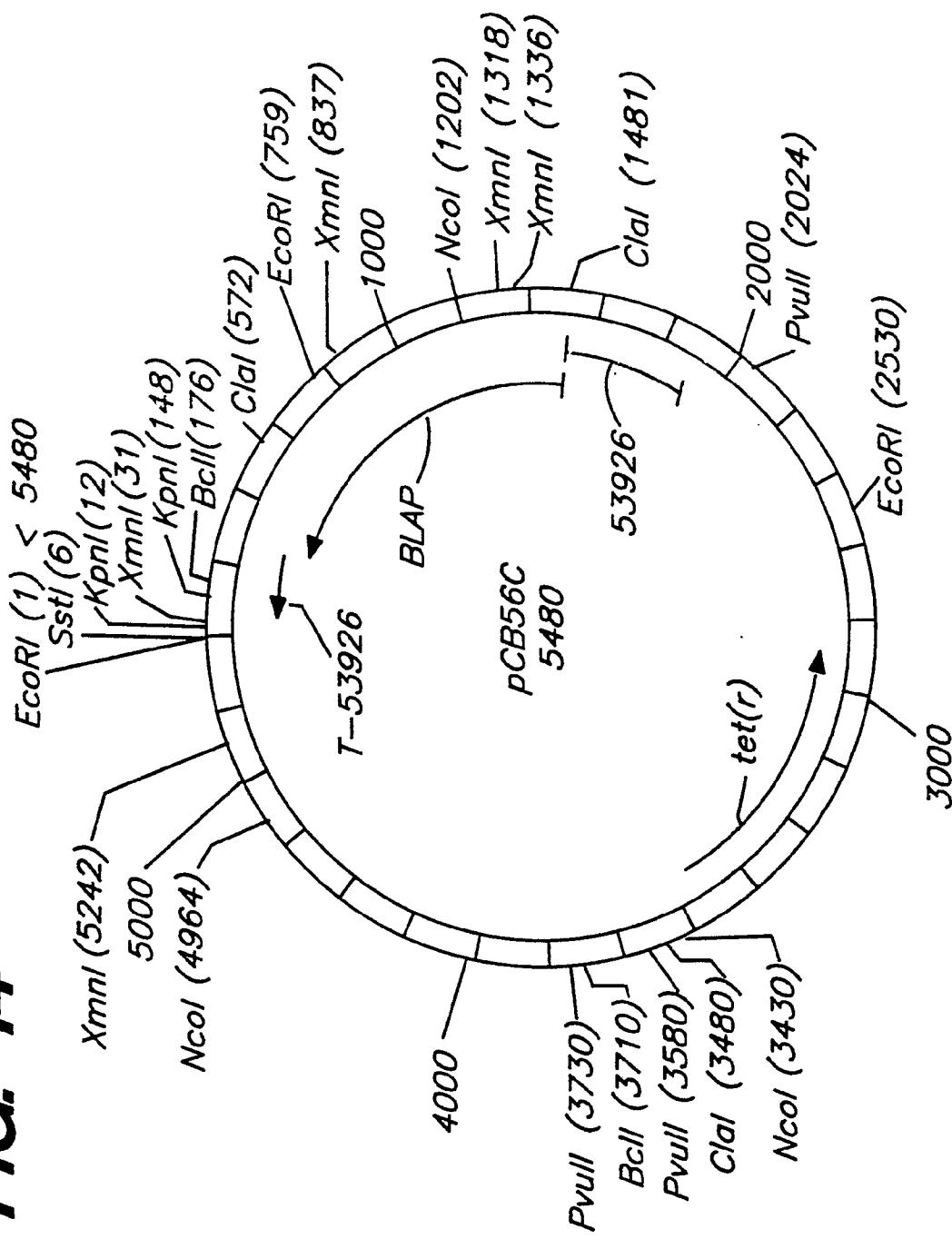
FIG. 15
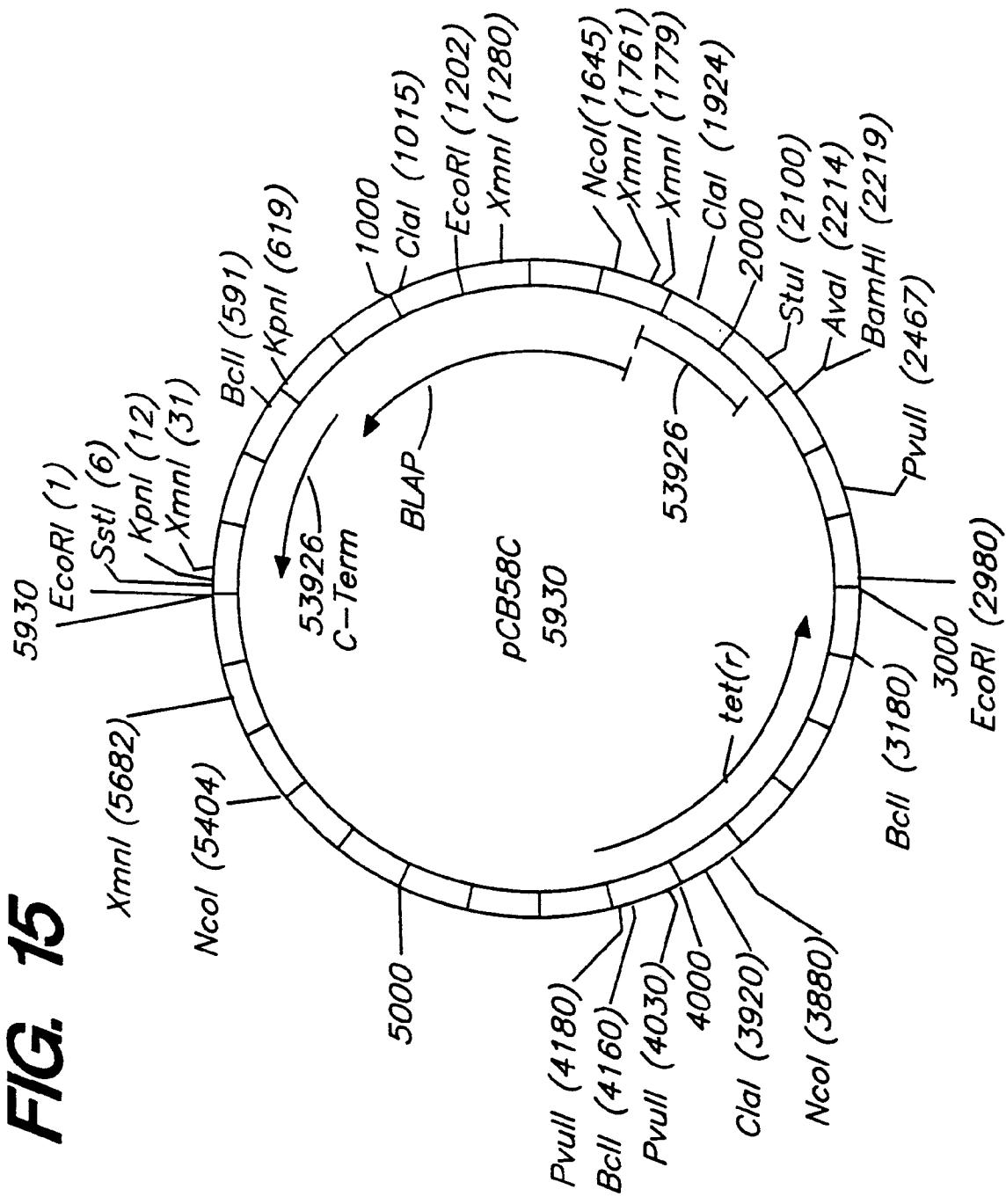
FIG. 16
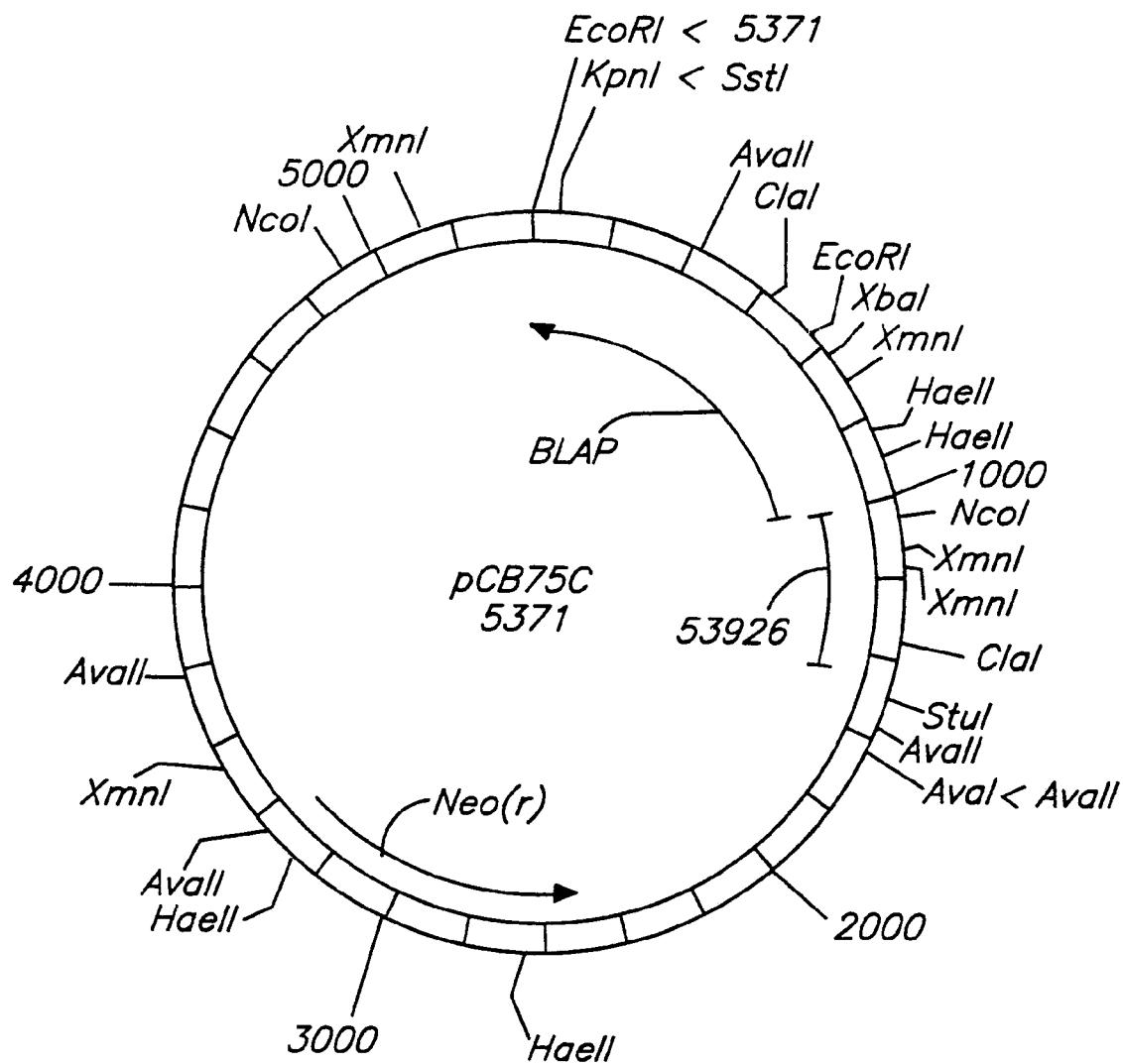
FIG. 17
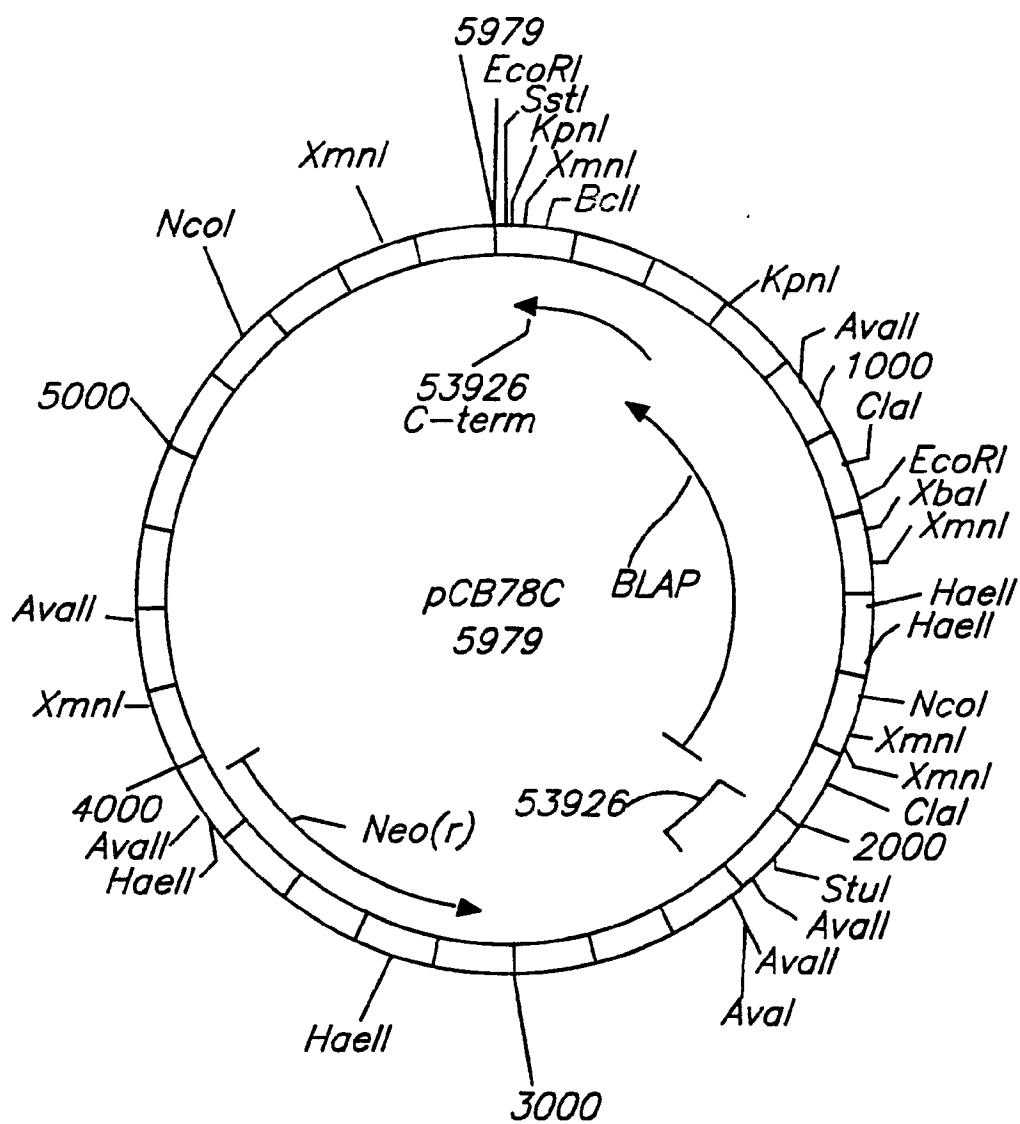
FIG. 18
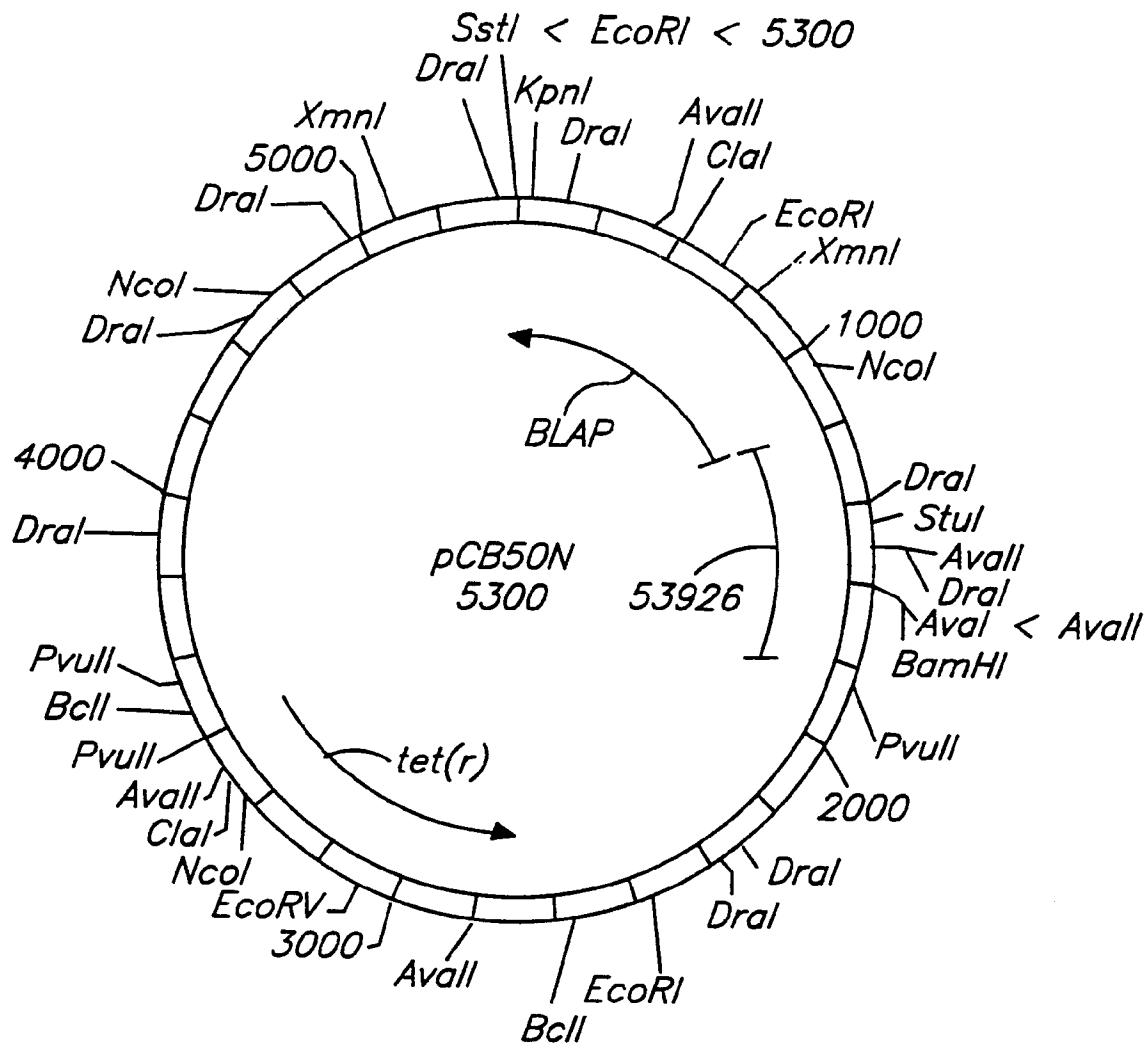
FIG. 19
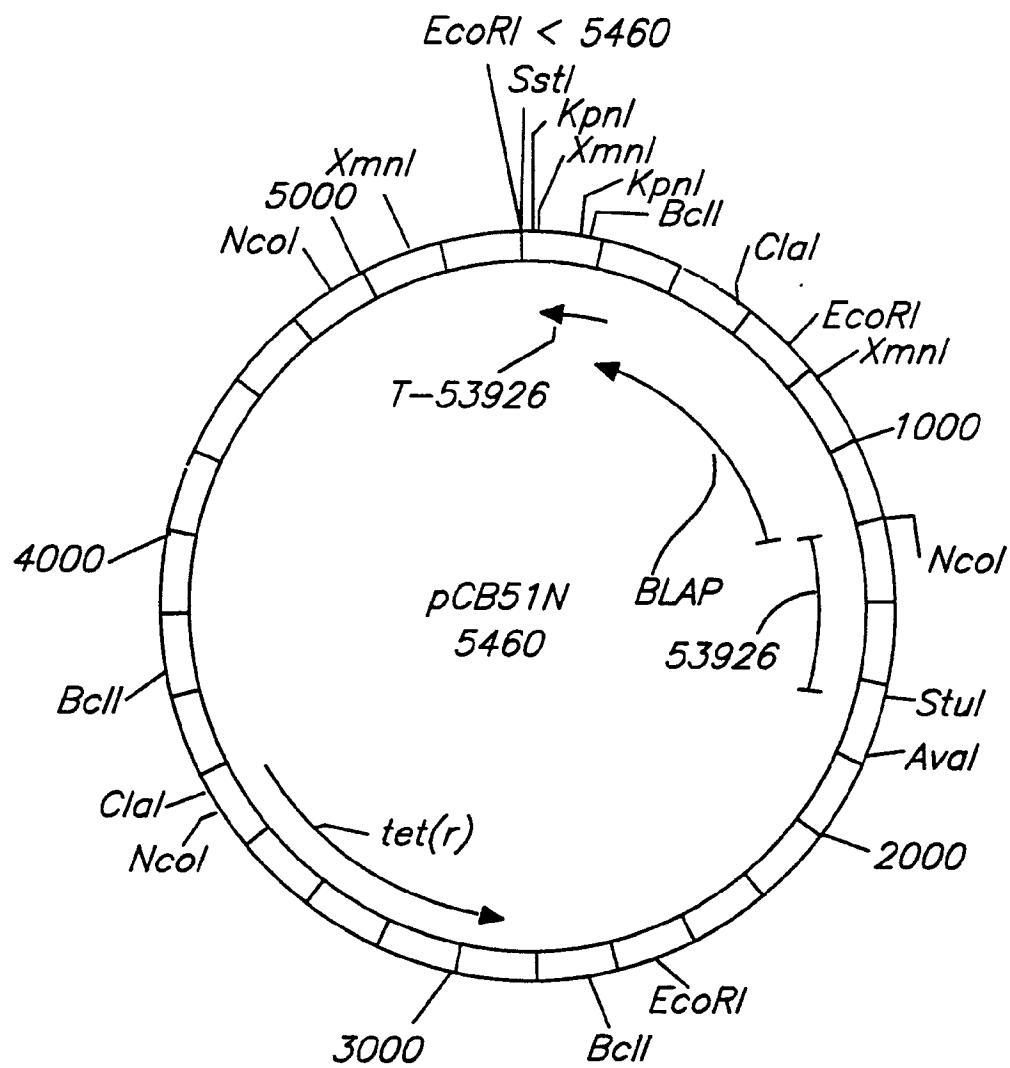
FIG. 20
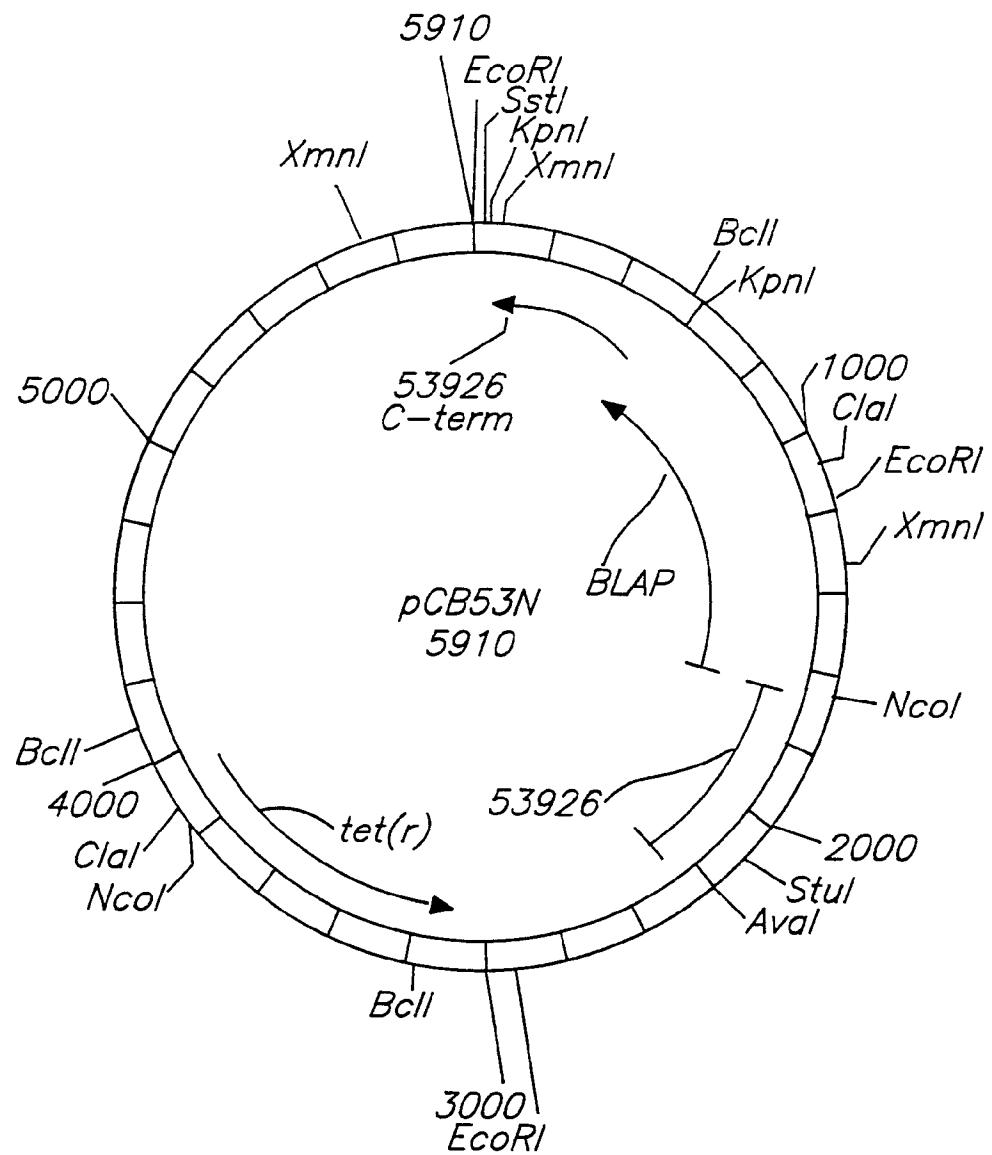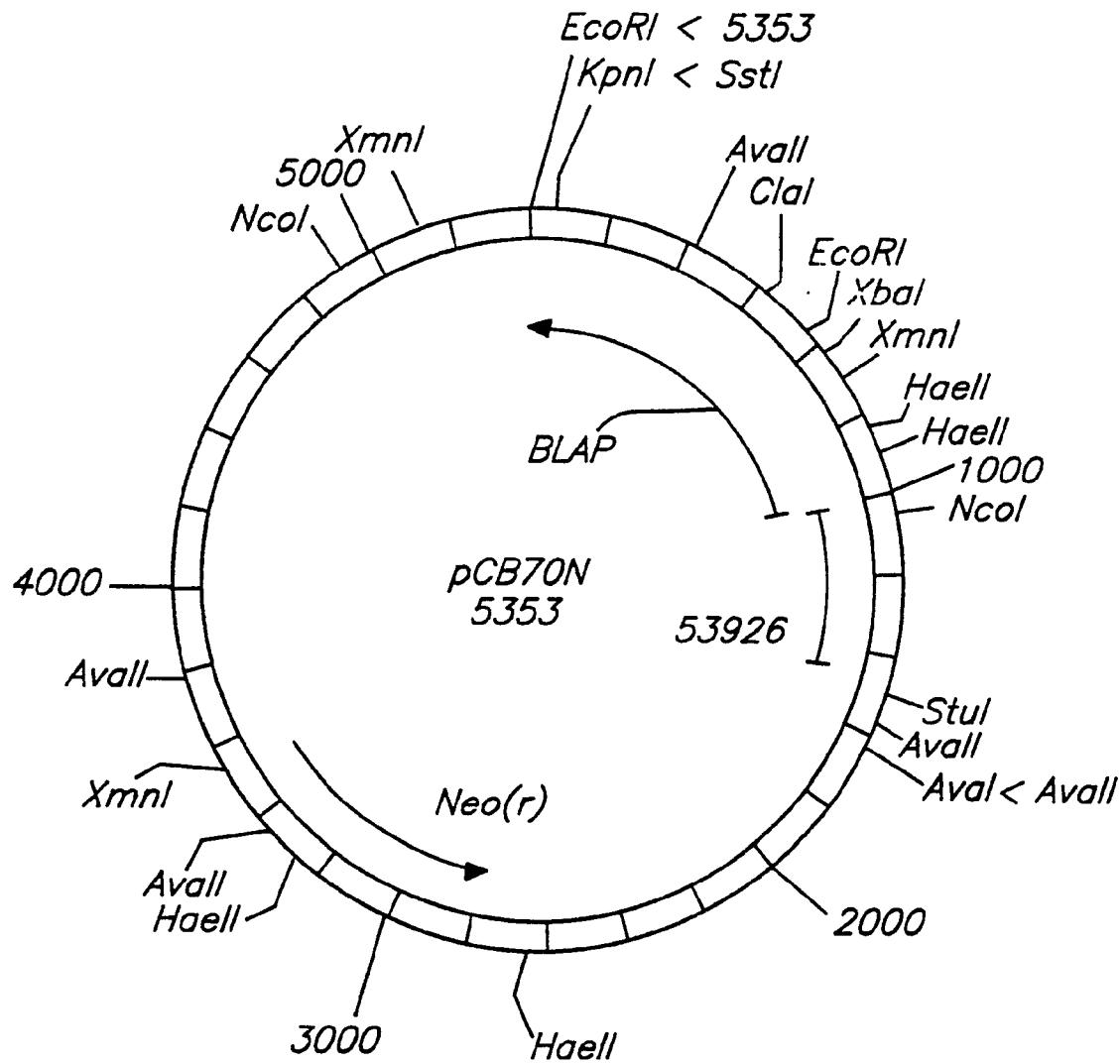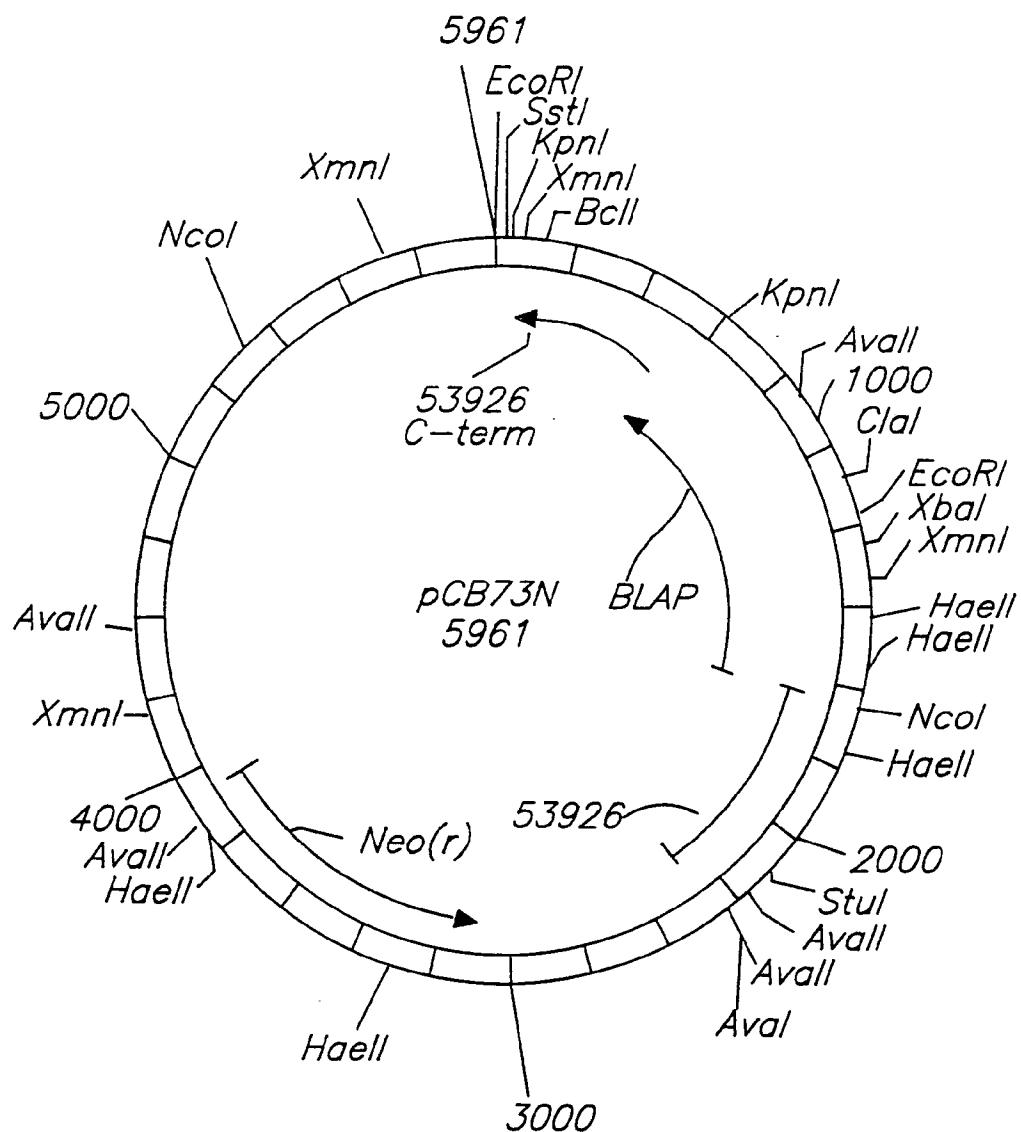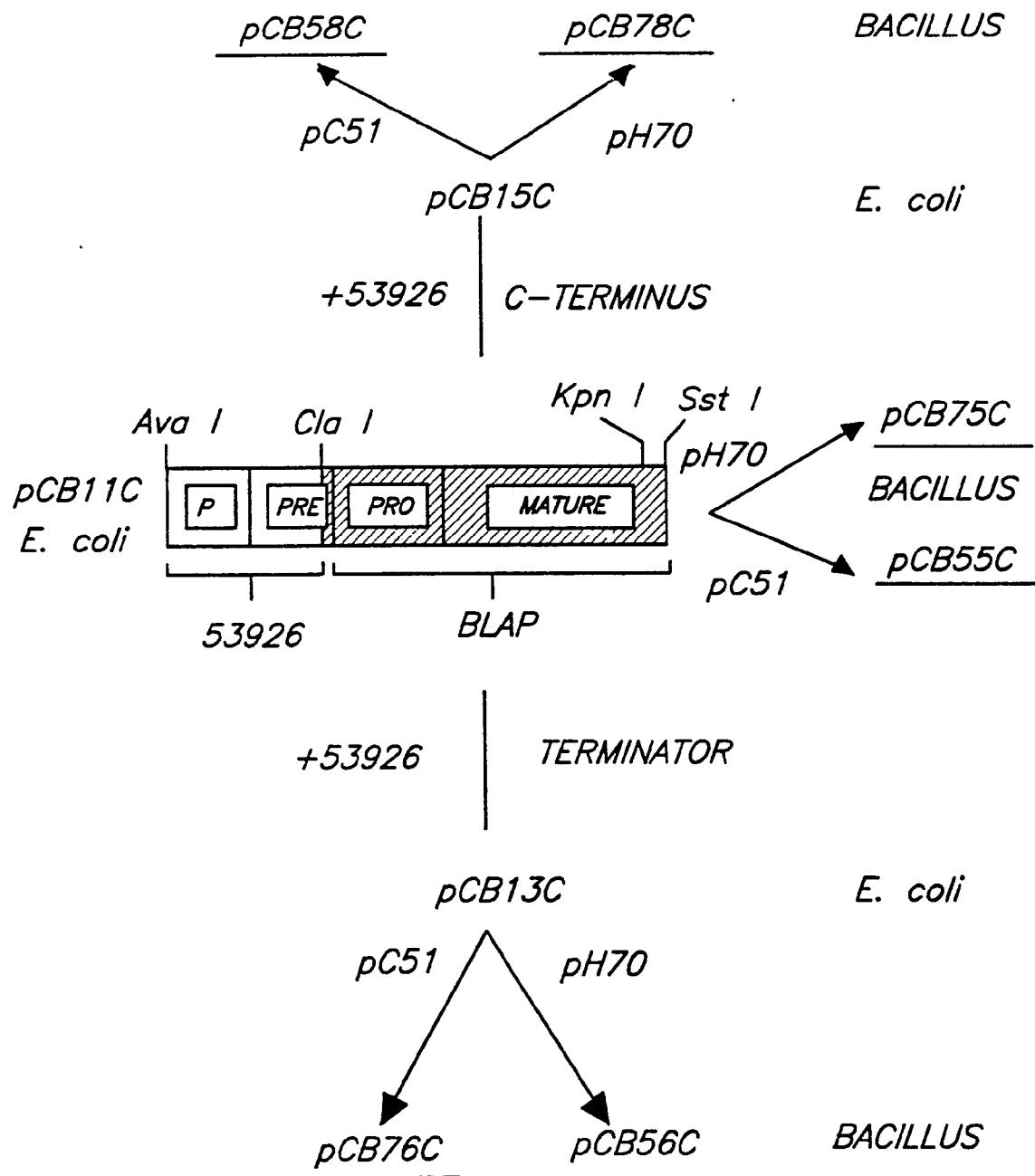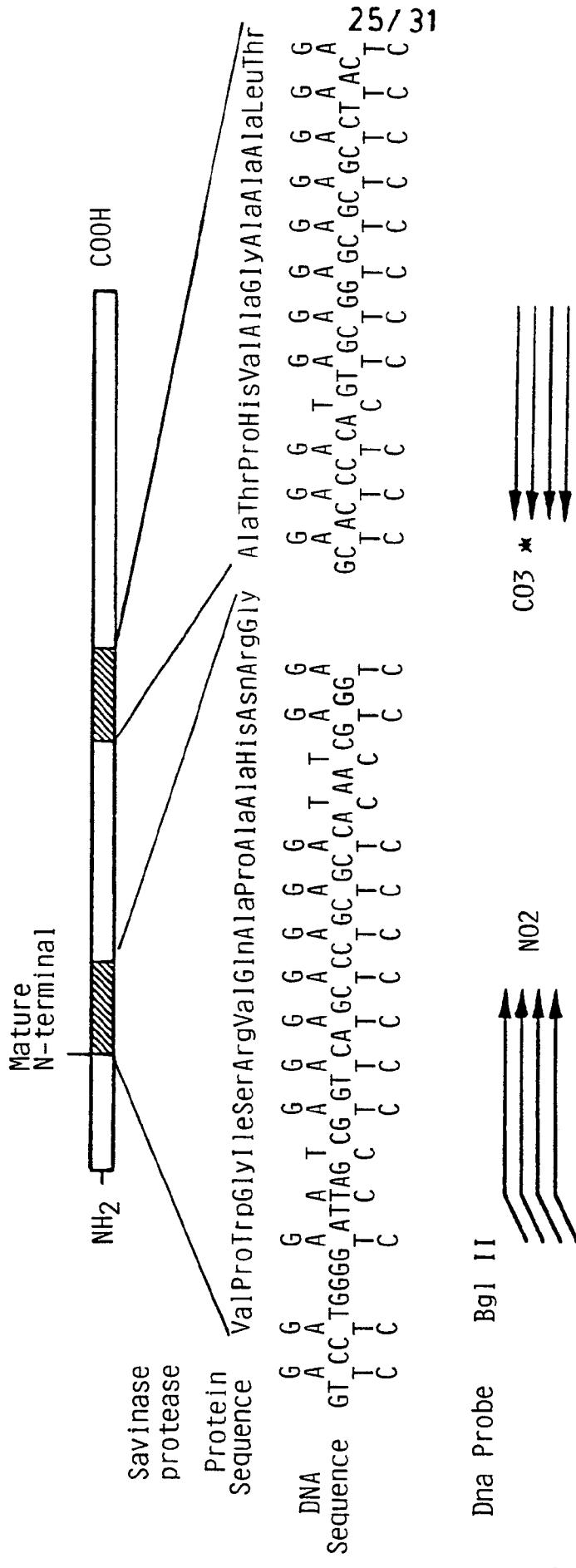
FIG. 21

FIG. 22

FIG. 23

FIG. 24

FIG. 25

```
NO2.1      5'    GGCAGATCTGTXCCXTGGGGGAT-3'
NO2.2      5'    GGCAGATCTGTXCCXTGGGGAAT-3'
NO2.3      5'    GGCAGATCTGTXCCXTGGGGTAT-3'
NO2.4      5'    GGCAGATCTGTXCCXTGGGGCAT-3'


                       A   T         T
NO4.1      5'    GTCCCGTGGGGXATCAG-3'
                                A
                       G   T         T
NO4.2      5'    GTTCCGTGGGGXATCAG-3'
                                A
                       A   T         T
NO4.3      5'    GTCCCGTGGGGXATCTC-3'
                                A
                       G   T         T
NO4.4      5'    GTTCCGTGGGGXATCTC-3'
                                A


                   A   A   A   A  T
CO3.1      5'    CCCGCCACGTGCGGCGT-3'

                   G   G   A   A  T
CO3.2      5'    CCTGCTACGTGCGGCGT-3'

                   G   A   A   A  T
CO3.3      5'    CCTGCCACGTGCGGCGT-3'

                   A   G   A   A  T
CO3.4      5'    CCCGCTACGTGCGGCGT-3'
```

X = (A,T,G or C)

FIG. 26

FIG. 27

```
          10        20        30        40        50
           |         |         |         |         |
  1  GTCTCCTGGATCTCATAAAATAAATGAATAGTATTTTCATAAAATGAATC

 51  ATATGGATGCAATCTCCTGTCATTCTGCTGGCCCTCGGGACCTCTTTCCC

101  TCGGAGGCTGAAGCGGTCTATTCATACTTTCGAACTGAACATTTTTCTAA

151  AACAGTTATTAATAACCAAAAAATTTTAAATTGGTCCTCCAAAAAAATAG

                                          TRANSCRIPTION START SITE
201  GCCTACCATATAATTCATTTTTTTTCTATAATAAATTAACAGAATAATTG

251  GAATAGATTATATTATCCTTCTATTTAAATTATTCTGAATAAAGAGGAGG

                        PRE-SEQUENCE
301  AGAGTGAGTAATGATGAGGAAAAGAGTTTTTGGCTTGGGATGCTGACGG

                            CG T                    PRO SEQUENCE
351  CCTTCATGCTCGTGTTCACGATGGCATTCAGCGATTCCGCTTCTGCTGCT
                            Cla I

401  CAACCGGCGAAAAATGTTGAAAAGGATTATATTGTCGGATTTAAGTCAGG

451  AGTGAAAACCGCATCTGTCAAAAAGGACATCATCAAAGAGAGCGGCGGAA

501  AAGTGGACAAGCAGTTTAGAATCATCAACGCGGCAAAAGCGAAGCTAGAC

551  AAAGAAGCGCTTAAGGAAGTCAAAAATGATCCGGATGTCGCTTATGTGGA

                              MATURE PROTEASE
                                       A GG
601  AGAGGATCATGTGGCCCATGCCTTGGCGCAAACCGTTCCTTACGGCATTC
                                      Nco I
651  CTCTCATTAAAGCGGACAAAGTGCAGGCTCAAGGCTTTAAGGGAGCGAAT

701  GTAAAAGTAGCCGTCCTGGATACAGGAATCCAAGCTTCTCATCCGGACTT

751  GAACGTAGTCGGCGGAGCAAGCTTTGTGGCTGGCGAAGCTTATAACACCG
```

801 ACGGCAACGGACACGGCACACATGTTGCCGGTACAGTAGCTGCGCTTGAC

851 AATACAACGGGTGTATTAGGCGTTGCGCCAAGCGTATCCTTGTACGCGGT

901 TAAAGTACTGAATTCAAGCGGAAGCGGATCATACAGCGGCATTGTAAGCG
        T C
951 GAATCGAGTGGGCGACAACAAACGGCATGGATGTTATCAATATGAGCCTT
      Kpn I
1001 GGGGGAGCATCAGGCTCGACAGCGATGAAACAGGCAGTCGACAATGCATA

1051 TGCAAGAGGGGTTGTCGTTGTAGCTGCAGCAGGGAACAGCGGATCTTCAG

1101 GAAACACGAATACAATTGGCTATCCTGCAAAATACGATTCTGTCATCGCT

1151 GTTGGTGCGGTAGACTCTAACAGCAACAGAGCTTCATTTTCCAGCGTCGG

1201 AGCAGAGCTTGAAGTCATGGCTCCTGGCGCAGGCGTATACAGCACTTACC

1251 CAACGAACACTTATGCAACATTGAACGGAACGTCAATGGCTTCTCCTCAT

1301 GTAGCGGGAGCAGCAGCTTTGATCTTGTCAAAACATCCGAACCTTTCAGC
                                                T C
1351 TTCACAAGTCCGCAACCGTCTCTCCAGCACGGCGACTTATTTGGGAAGCT
                                                  Kpn I
1401 CCTTCTACTATGGGAAAGGTCTGATCAATGTCGAAGCTGCCGCTCAATAA

1451 CATATTCTAACAAATAGCATATAGAAAAAGCTAGTGTTTTTAGCACTAGC

1501 TTTTTCTTCATTCTGATGAAGGTTGTTCAATATTTTGAATCCGTTCCATG
        G AC
1551 ATCGTCGGATGGCCGTATTTAAAAATCTTGACGAGAAACGGCGGGTTTGC
        Kpn I
1601 CTCGCTCAGCCCGGCTTTTGAGAGCTCTTGAAACGTCGAAACCGCTGCAT

1651 CGCTGTTTTGCGTCAGTTCAATCGCATACTGGTCAGCAGCTTTTCCTGAT

1701 GCCTGAAACTGCTGCTAAATGAGACGA   **FIG. 27A**

FIG. 28
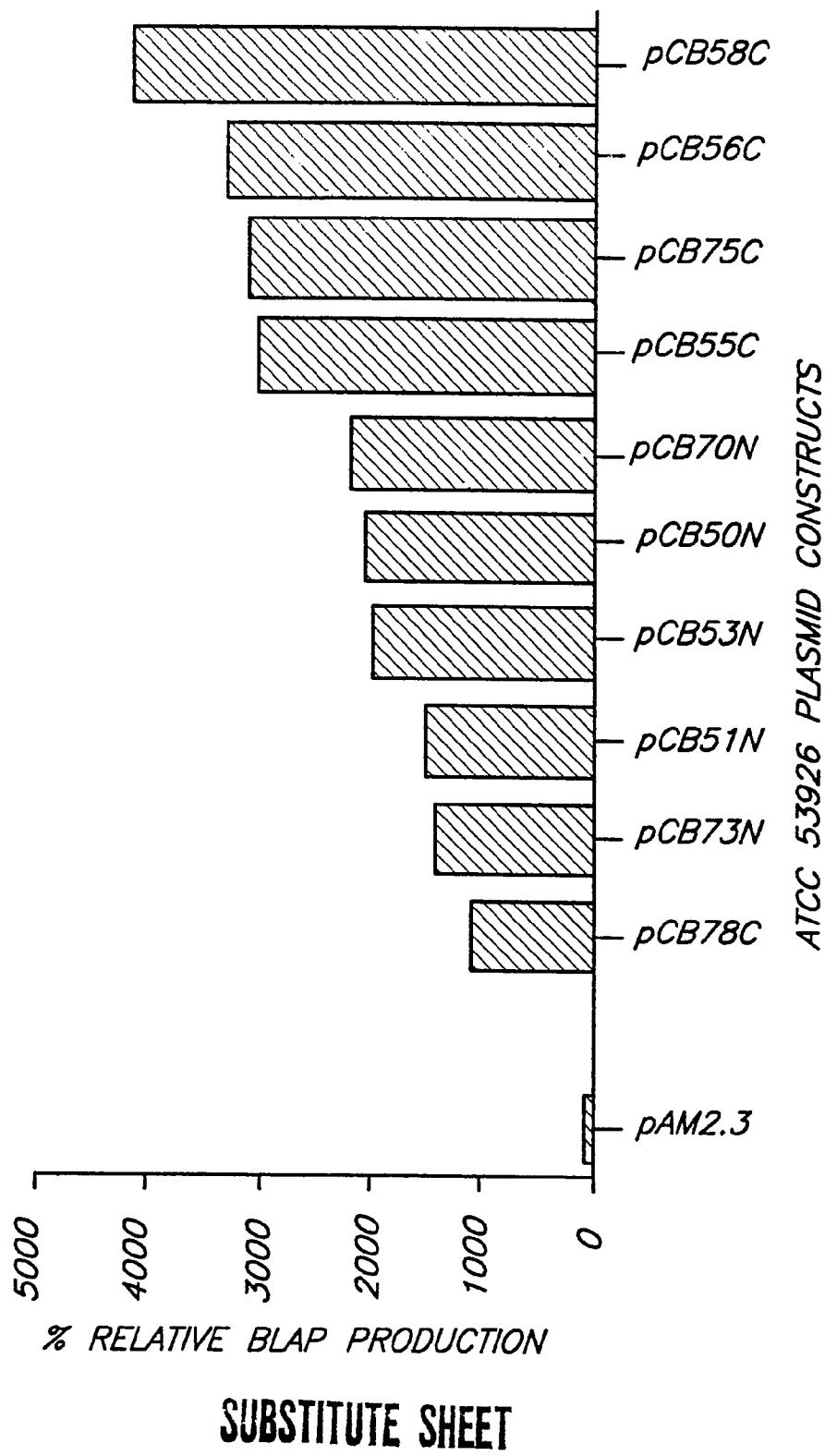
```
                5         10        15        20        25        30
                |         |         |         |         |         |
     1  A Q T V P W G I S R V Q A P A A H N R G L T G S G V K V A V
    31  L D T G I S T H P D L N I R G G A S F V P G E P S T Q D G N
    61  G H G T H V A G T I A A L N N S I G V L G V A P S A E L Y A
    91  V K V L G A D G R G A I S S I A Q G L E W A G N N G M H V A
   121  N L S L G S P S P S A T L E Q A V N S A T S R G V L V V A A
   151  S G N S G A S S I S Y P A R Y A N A M A V G A T D Q N N N R
   181  A S F S Q Y G A G L D I V A P G V N V Q S T Y P G S T Y A S
   211  L N G T S M A T P H V A G A A A L V K Q K N P S W S N V Q I
   241  R N H L K N T A T S L G S T N L Y G S G L V N A E A A T R
```

Amino acid composition

| 41 A	0 C	7 H	3 M	18 T
9 R	10 Q	10 I	2 F	3 W
22 N	5 E	19 L	13 P	7 Y
6 D	34 G	5 K	31 S	24 V

Number of residues: 269
Molecular weight (MW): 26837

FIG. 29

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/04673

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^5: C 12 N 9/54, 15/57, 15/75, C 11 D 3/386, //(C 12 N 1/21,
C 12 R 1:10)(C 12 N 1/20, C 12 R 1:07, 1:10)

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^5	C 12 N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	WO, A, 89/06279 (NOVO INDUSTRI A/S) 13 July 1989 see abstract; table 1a,; page 14, line 20 - page 17, line 24	1,36,47
A	--	2-5,16-25
Y	EP, A, 0214435 (HENKEL KOMMANDITGESELL- SCHAFT AUF AKTIEN) 18 March 1987 see abstract; page 2, lines 10-15; all claims	1,36,47
A	--	2-4,16-25
A	GB, A, 2171703 (AGENCY OF INDUSTRIAL SCIENCE AND TECHNOLOGY) 3 September 1986 see abstract	2-4

----

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26th Oktober 1990	17. 12. 90

International Searching Authority	Signature of Authorized Officer
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